ISOLATION AND CHARACTERIZATION OF A TRANSCRIPTION PRE-INITIATION COMPLEX COMPONENT, FACTOR IIB, FROM PLANTS

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LIST OF ABBREVIATIONS

A anisotropy
AD activation domain
AdMLP adenovirus major late promoter
β-gal beta-galactosidase
bp base pair
BSA bovine serum albumin
bZIP basic region/leucine zipper
CaMV cauliflower mosaic virus
CBP CREB binding protein
CMV cytomegalovirus
cpm counts per minute
CREB cAMP response element binding
CTAB cetyltrimethylammonium bromide
CTD carboxy-terminal domain
DBD DNA-binding domain
EMSA electrophoretic mobility shift assay
5-FOA 5-fluoroorotic acid
Ftz fushi taratzu
GS4b glutathione-Sepharose resin 4b
GST glutathione S-transferase
HeLa a human cancer cell culture
hr hour
I intensity of fluorescence
Inr initiator element
IPTG isopropyl β-D-thiogalactoside
k_a rate of association
k_d rate of dissociation
K_d equilibrium dissociation constant
kDa kilodalton
μg microgram
min minute
ml mililiter
mM milimolar
NF-κB nuclear factor kappa B
ng nanogram
nm nanometer
nM nanomolar
P polarization
PCR polymerase chain reaction
PIC pre-initiation complex
pmol picomole
pol II RNA polymerase II
RAP RNA pol II associated protein
SDS sodium dodecyl sulfate
sec second
SGBF2 soybean G-box binding factor 2
TAF TBP-associated factor
TBP TATA-binding protein
TFIIB transcription factor for pol II B
TRα, β thyroid hormone receptor α or β
TX100 TRITON X-100
VDR vitamin D receptor
VP1 viviparous 1
VP16 herpes virus viral protein 16

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Initiation of eukaryotic transcription requires formation of a pre-initiation complex which contains multiple protein components that direct RNA polymerase II to promoter DNA sequences and converts the enzyme to a mode competent for transcription. While most of the proteins contained in this complex have been identified in animal, insect and yeast systems, clones for the corresponding plant genes have generally not been characterized. This work reports the isolation of cDNA clones for transcription factor IIB (TFIIB) from *Arabidopsis thaliana* and soybean. TFIIB is known to play an important part in early events leading to formation of the pre-initiation complex and, therefore, may have roles as a target for regulation of transcription as well as a bridge between regulatory proteins, complex components, and RNA polymerase II. Sequence comparisons between the plant TFIIB clones and versions from other species show a high degree of homology and suggest significant functional conservation. An investigation of the functional properties of the *Arabidopsis* TFIIB protein found that this factor could replace its human counterpart in a HeLa *in vitro* transcription assay.

Additional *in vitro* experiments were conducted to measure TFIIB binding affinity for another plant pre-initiation complex member, the TATA-binding protein, and DNA. *Arabidopsis* TFIIB had an equilibrium binding constant of 7.1 nM for formation of this ternary complex and was also capable of associating with TATA-binding proteins from humans and yeast. The observed inter-species binding failed to predict ability to substitute in transcription, however, as replacement of the yeast homolog with *Arabidopsis* TFIIB produced no viable cells. Many transcriptional regulators have binding affinity for TFIIB, and this has led to the hypothesis that the protein is a target of regulatory mechanisms. *Arabidopsis* TFIIB was shown to bind to the herpes virus VP16 acidic domains, the maize acidic activator VP1, and a soybean proline-rich transcription factor SGBF2. TFIIB binding reactions with all three factors were not affected by high salt concentrations, but could be disrupted to various degrees by detergents. The demonstration that core regions of *Arabidopsis* TFIIB are involved in these interactions is consistent with similar mapping experiments for TFIIB homologs from other species.

CHAPTER 1
INTRODUCTION

The Pre-Initiation Complex of RNA Polymerase II

Eukaryotic RNA polymerase II (pol II) is the nuclear enzyme which transcribes DNA to RNA for genes encoding proteins, and consists of 8-14 subunits with total mass greater than 500 kilodaltons (kDa). Despite this complexity, the enzyme has no inherent ability to initiate transcription in a gene-specific manner. This is the function of a second multi-subunit complex, containing at least 25 polypeptides and a mass over 750 kDa, which assembles on the DNA template within the promoter region and at the start site for transcription. The pre-initiation complex (PIC) contains both sequence-specific and non-specific DNA-binding proteins as well as many factors that do not contact DNA. Interactions among these factors and with pol II localize the polymerase to the start site, and enzymatic activities within the PIC then modify pol II to an active state and open the template double helix for RNA initiation.

Chromatographic separation of PIC components necessary for *in vitro* transcription established the order of assembly: fraction D (TFIID, transcription factor for pol II), which binds first, contains the TATA-binding protein (TBP) and its associated factors, followed by TFIIA, TFIIB, pol II, the two proteins in TFIIF, and factors in fractions E and H (Buratowski *et al.*, 1989; Flores *et al.*, 1991; Flores *et al.*, 1992; Killeen and Greenblatt, 1992; Maldonado *et al.*, 1990). X-ray crystallography of TBP (Nikolov *et*

al., 1992) reveals a saddle shaped structure which binds in a sequence specific manner to the DNA minor groove of the TATA box, an element commonly found in pol II promoters around 25 base pairs (bp) upstream of the start site. Mutation of the TATA box sequence in some instances can prevent TBP binding without repressing transcription. These experiments often show an alteration in the position of the start site, and when combined with the observation that some promoters do not contain a TATA box, led to the characterization of the initiator (Inr) element (reviewed in Kraus *et al.*, 1996). This sequence, present in TATA-less and some TATA-containing promoters, is located around the start site and directs formation of the PIC without sequence-specific binding of TBP. The functional flexibility of TBP reflects its utilization in PICs for RNA polymerases I and III; much of this plasticity may be due to the TBP associated factors (TAFs) which co-purify with TBP in fraction D. While the composition varies among species, the TAFs generally comprise a group of around ten proteins ranging from 18 to 250 kDa, and their presence in *in vitro* footprinting reactions with TBP extends the protected region upstream of the TATA box and downstream past the start site (Verrijzer and Tjian, 1996). When only an Inr element is present, the TAFs are necessary for formation of the DNA-TFIID complex. Sub-stoichiometric levels of some TAFs in TFIID compared to TBP suggest there may be several versions of the TBP-TAF complex *in vivo*, and these could generate specificity for different promoter architectures within and among the three polymerase systems.

TFIIA contains two or three proteins (Roeder, 1991) that associate with the N-terminal stirrup of TBP and with DNA, thus clamping TBP to its binding site and perhaps stabilizing the interaction by counteracting negative factors in TFIID (Triezenberg, 1995).

The presence of TFIIA is not always required for *in vitro* transcription and further study is needed to completely define its role. TFIIA stabilization of TBP may only be necessary in certain promoter or TAF contexts, or in instances where other regulatory factors exert their influence on transcription via TFIIA interaction. Like TFIIA, TFIIB clamps TBP to the promoter but through association with the C-terminal stirrup, stabilizing the interaction on the opposite DNA face. Unlike TFIIA, factor B is a single 35 kDa polypeptide required for all pol II transcription. Alone, TFIIB has no affinity for DNA but when bound to the TATA-TBP complex there are contacts with the phosphodiester backbone as revealed by the crystal structure of the ternary complex (Nikolov *et al.*, 1995). TBP places a severe kink in the DNA helix, and in the presence of TFIIA and TAFs, some of which contain histone-like domains, the proximal promoter region seems to be wrapped around the nascent PIC, positioning the start site near the region where TFIIB binds TBP (Oelgeschlager *et al.*, 1996). This correlates with previous observations that mutations in TFIIB can alter the position of the start site and that TFIIB may provide a bridging function between the TATA region and pol II.

RNA polymerase II has high *in vitro* binding affinity for preformed DNA:TFIID:TFIIB (DB) or DAB complexes, establishing a closed promoter complex, but conversion to the open promoter form required for transcription initiation does not proceed without factors from fractions F, E, and H. TFIIF contains two polypeptides, RAP30 and RAP74 (Greenblatt, 1991), which associate as a complex that binds pol II before entry into the PIC. RAP30 has a region of homology to the bacterial transcription factor sigma 70 as well as a DNA binding domain (Tan *et al.*, 1994), features consistent with one of the proposed roles for TFIIF as an inhibitor of non-specific DNA binding by

pol II and a primary site of interaction between the polymerase and the PIC-DNA. RAP74 can be photocross-linked with template nucleotides adjacent and upstream to the start site (Robert *et al.*, 1996) and may promote melting of the double stranded DNA prior to RNA initiation (Pan and Greenblatt, 1994; Robert *et al.*, 1996). De-phosphorylation of pol II is also influenced by RAP74. The largest subunit of pol II contains a number of tandem repeats forming a region called the carboxy-terminal domain (CTD) which associates with multiple members of the PIC and other transcription factors (holoenzyme components, described below) when unphosphorylated (Emili and Ingles, 1995; Lu *et al.*, 1991). Disruption of this binding, especially to TBP, requires extensive phosphorylation by a CTD kinase in the assembled PIC and is necessary for release of pol II from the promoter and elongation of the transcript (Laybourn and Dahmus, 1990). CTD phosphatase activity occurs, presumably after termination of transcription, to restore the CTD to a form competent for entry into another PIC. The phosphatase apparently docks to a site on pol II other than the CTD, and its activity is stimulated by RAP74 (Chambers *et al.*, 1995). The same study suggested that this reaction may be inhibited by TFIIB after pol II entry into the PIC.

The CTD kinase is thought to be a part of TFIIH, a nine subunit complex that contains DNA-dependent ATPase-helicases and a cyclin-dependent kinase activating kinase activity (Feaver *et al.*, 1994). Pol II phosphorylation is probably a progressive event, with initial modification of the hypophosphorylated CTD (Laybourn and Dahmus, 1990) resulting in reduced affinity for the PIC and later kinase activity converting the enzyme to the form required for efficient elongation; this progression can be blocked *in vivo* by anti-TFIIH antibodies and kinase inhibitors which specifically inhibit elongation

but not initiation (Yankulov *et al.*, 1996). The helicase activities of TFIIH, along with TFIIE, are required for formation of the open promoter complex in a two-step process that can be distinguished by changes in DNA topology. Potassium permanganate probing of single-stranded thymidines indicates ATP-dependent melting of the region –9 to +1 nucleotides (relative to the start site) before initiation. After formation of the first RNA phosphodiester bond, the single-stranded region expands to +8 (Holstege *et al.*, 1996). The initiation process is complete upon promoter clearance by pol II, but the enzyme in some cases becomes stalled after incorporating 10-30 nucleotides of RNA (O'Brien *et al.*, 1994). TFIIF remains associated with pol II and may, in conjunction with other factors, assist in the conversion to full elongation mode (Chang *et al.*, 1993). All the other components of the PIC, except the factors in TFIID, dissociate from the promoter, leaving only the DNA-TBP:TAF complex as a starting point for re-initiation (Zawel *et al.*, 1995).

Much of the work described above characterized transcription *in vitro*, allowing careful dissection of the factors necessary for pol II initiation. These systems, however, suffered from an inability to boost production of RNA transcripts in the presence of activator proteins known to enhance transcription by binding to promoter regions upstream of the TATA box. Clearly, the general factors could be assembled for a basal level of transcription but other proteins required for enhanced RNA production, or activated transcription, were missing. Two lines of research in yeast have provided further insight. Partial deletion of the CTD produces several conditional mutant phenotypes that can be overcome by four different dominant suppressors. These proteins, the SRBs (suppressor of RNA polymerase B, Koleske and Young, 1994), bind to the CTD and co-purify with a large complex containing pol II and many of the general

factors, including TFIIB but not TFIID, which are pre-associated with the enzyme despite the absence of promoter DNA. The SRBs were also present in an enhanced complex of pol II isolated by purification of a "mediator" fraction that supported activated transcription (Kim *et al.*, 1994). This form of pol II, termed the holoenzyme, additionally contains other proteins previously known to be important for transcriptional regulation, including the SWI/SNF complex which disrupts nucleosome structure to open chromatin for promoter accessibility (Wilson *et al.*, 1996). The combination of gentle cell disruption and affinity purification or immuno-precipitation to isolate the yeast holoenzyme was successfully used to prepare a similar holoenzyme from rat liver nuclear extracts (Ossipow *et al.*, 1995). This preparation was initially thought to contain all the general PIC factors, but refinement of the technique suggests TFIID and TFIIB are not part of the mammalian holoenzyme (Cujec *et al.*, 1997).

Regulation of Transcription

The discovery of the holoenzyme is one turning point in a trend to refine the model of the PIC, especially as it relates to regulation of transcription. Many regulatory factors that bind to upstream promoter elements have *in vitro* binding affinity for members of the PIC, and an ordered progression of PIC assembly easily fit into a model whereby different regulators influence each step. Recruitment by an upstream factor would increase the local concentration of a PIC component, thus accelerating the rate of assembly, and the presence of more factors recruiting many different parts of the PIC would give correspondingly higher levels of transcription. A pre-assembled holoenzyme, however, arrives in one binding event and does not require stepwise recruitment of its

components to build the PIC. Regulation of transcription may therefore involve potential cooperative interactions with multiple targets in the holoenzyme, rather than progressive PIC assembly. Promoters usually contain binding sites for several transcription factors and combinations of these factors often have a synergistic effect, which may be a result of coopertivity in binding. Interactions between transcription factors and their targets are thought to be highly cooperative since both components also have affinity for the promoter, so multiple links between the holoenzyme and the promoter may represent a synergistic combination of such interactions.

As models for transcriptional regulation are refined, greater emphasis is being placed on what mechanisms, in addition to recruitment of basal factors, might influence the various interactions observed among PIC members. The research to understand these mechanisms continues to benefit from new additions to both categories of interactants involved. The targets of regulation are being identified as proteins in the classical PIC or holoenzyme, and the upstream regulators are being characterized as members of groups with shared functions and/or sequence homologies. The following sections summarize these functional classes and discuss some of the steps at which they may act.

Activators

Transcriptional activators are proteins that exhibit DNA-binding affinity and increase levels of transcription as a consequence of their DNA association. Indirect activators such as HMG-2 (Shykind *et al.*, 1995) show broad sequence specificity and remodel chromatin in distal promoter regions to expose binding sites for other factors or serve as architectural proteins to stabilize DNA conformation at the PIC. Other indirect activators recognize specific cis-elements within a promoter but require dimerization with

a second protein to activate transcription (see Co-activators), or distort the DNA to help bring upstream sites nearer the PIC as is the case with the CCAAT-box binding protein NF-Y (Ronchi *et al.*, 1995).

Direct activators combine DNA binding domains with one or more protein interaction motifs that influence transcription. The DNA elements to which they localize may be near the site of PIC formation or quite far (>1 kb) upstream from the TATA region, and may even be found following the 3' end of a gene's coding sequence. The DNA looping required to bring these element-activator complexes into the vicinity of the PIC has been visualized by atomic force microscopy (Becker *et al.*, 1995) and often requires stabilization by other factors and cooperative interactions at both the DNA binding site and to the PIC target. Distal cis-elements in some cases may also utilize specific spacing patterns relative to nucleosome structure for positioning near the TATA region.

The protein interaction motifs that confer an ability to boost transcription are termed activation domains (AD). These peptide sequences are usually separated from the DNA-binding domain (DBD) and can be isolated and studied as fusions with a well-characterized DBD to facilitate both *in vitro* and *in vivo* functional mapping. Conservation among some ADs can be observed as a general enrichment for particular amino acids such as proline, serine or glutamine, or at the level of residue organization (reviewed in Triezenberg, 1995). Acidic activators, for example, often contain regions high in glutamic and aspartic acids divided into blocks of conserved length by bulky hydrophobic residues. The term "acidic activators" is also an example of the potential pitfalls of AD classification by amino acid composition. Several mutational analyses

have made large scale charge alterations and observed little effect on transcriptional activation. Changing particular hydrophobic residues or the patterning of aromatic and hydrophobic residues is more likely to disrupt activation potential (Jackson *et al.*, 1996; Regier *et al.*, 1993; Sainz *et al.*, 1997).

Secondary structure has been difficult to detect for many ADs, leaving most to be classified as random coils, unstructured, or acid "blobs." Examples are known which contain alpha-helices, beta-strands, Zn fingers and leucine zippers, and secondary structure can sometimes be induced under special conditions (Shen *et al.*, 1996). The c-myc transactivation domain has no secondary structure when observed by circular dichroism in aqueous buffer, but measurements in hydrophobic solvent or in the presence of TBP suggest an alpha-helical conformation (McEwan *et al.*, 1996). Even when unstructured, many ADs can be functionally divided into subdomains that correspond to distinct patterns of residue predominance or charge, and these subdomains have often been shown to make synergistic contributions to transcription activation (Artandi *et al.*, 1995; Blair *et al.*, 1996).

Co-activators

As clones for activators were isolated, a common method for studying their effects utilized over-expression of the protein and measurement of transcription from a reporter gene whose promoter featured multiple binding sites for the activator. In some cases, over-expression led to repressed levels of transcription rather than activation. This inhibition of activity, or squelching, was thought to be due to depletion of cofactors by excess activator not bound to DNA (Gill and Ptashne, 1988; Martin *et al.*, 1990; Meyer *et al.*, 1989). Subsequent purification by activator affinity chromatography identified co-

activators as factors capable of increasing transcription by acting as adapters between DNA-bound regulators and the PIC. Cellular co-activators can have broad specificity for enhancement of several classes of activation domains, or can be limited to a narrow subset of promoter interactions. For example, human PC4 is a serine-rich co-activator that interacts with representatives of the acidic, proline, and glutamine classes of activators, and targets TFIIA in a phosphorylation-dependent manner (Ge and Roeder, 1994). In contrast, the human thyroid hormone receptor α (TRα) forms a complex with specific co-activators only in response to binding of the hormone ligand (Fondell *et al.*, 1996). Several members of the PIC also fit the definition of co-activators. Some TAFs and various holoenzyme components such as the mediator fraction are dispensable for basal transcription but are required for activation by upstream factors.

Viruses seem to be a rich source of co-activators. The adenovirus E1A factor has no DBD but activates a number of promoters, both cellular and viral, and contains a zinc binding domain that interacts with TBP or TAFs (Folkers and van der Saag, 1995). Herpes simplex viral protein VP16 is a common model for acidic activation domains, two of which are located in the C-terminal 80 amino acids. VP16 activates transcription of the viral immediate early genes by interacting with the cellular factor Oct-1, which recognizes binding sites within these viral promoters. The protein arrives as a part of the virion tegument (Elliott *et al.*, 1995) and, after nuclear localization, activates transcription through one or more interactions with TFIIB, TBP, TAFs, TFIIH, and PC4 (reviewed in Ghosh *et al.*, 1996). In addition to this unusually large number of potential targets for direct activation, as measured by *in vitro* binding affinity, VP16 has an anti-repressor

activity that epitope maps outside the acidic domains and may be due to dislocation of the histone H1 subunit (Lyons and Chambon, 1995).

Repressors

The number of identified transcriptional repressors continues to rapidly increase, and regulation by repression may be as important a mechanism as activator-dependent promoter control. Repressors exhibit the same range of interactions as activators and can act not just to prevent activated transcription but also to suppress promoters below their normal basal level. Unliganded TRα occupies its normal promoter cis-element and prevents transcription by direct contact with TBP, preventing entry of TFIIA or B into the PIC (Fondell *et al.*, 1996). Repression is relieved upon hormone binding; the ligand-binding domain is also the region contacting TBP for repression. E1A from adenovirus has a repression domain, distinct from the co-activation region, which also binds TBP and prevents transcription from some cellular promoters. Repression can be overcome *in vitro* by adding extra TF1IB, suggesting the E1a repressor blocks TBP-TF1IB interaction (Song *et al.*, 1997). Other repressors target TFIIB directly, TFIIA, or pol II itself (Gu *et al.*, 1995; Kirov *et al.*, 1996; Lee *et al.*, 1996). Some repressors are bi-functional and activate transcription when bound to an upstream location within a promoter but repress when located near the TATA box (Dostatni *et al.*, 1991). Conversely, the *Drosophila* Kruppel protein binds as a monomer to TATA proximal sites and activates through TFIIB contacts, but as a dimer binds to distal sites and represses transcription through TF1IE interactions (Sauer *et al.*, 1995). Regulators may actively repress by protein-protein interaction with PIC components, or cause passive repression by blocking a DNA sequence normally bound by an activator (Mailly *et al.*, 1996).

Squelching experiments similar to those for co-activators have identified co-repressors. Over-expression of the N-terminal half of the ligand binding domain from thyroid hormone receptor β prevents repression by the wild type factor, presumably by depleting a necessary co-repressor (Nawaz *et al.*, 1995; Tong *et al.*, 1996). Homologs of the Dr1 protein from yeast and HeLa cells are DNA-binding repressors that can be counteracted by acidic or glutamine-rich activators, but not by proline domain activators. Dr1 repression is boosted by heterodimerization with a co-repressor, DRAP1, which increases Dr1 affinity for TBP at the TATA box and blocks TFIIA and TFIIB binding (Mermelstein *et al.*, 1996). The repression domain of DRAP1 is proline-rich, and that of Dr1 contains multiple glutamine and alanine residues.

Other mechanisms for repressing transcription do not involve separate repressor proteins as described above but should be noted. The simple lack of an activator, perhaps by increased proteolysis or down-regulated expression of its promoter, is undoubtedly a very common method of gene repression, as is silencing by chromatin structure and promoter methylation. Some activators contain intrapeptide repression domains. Examples include heat shock transcription factors (Bonner *et al.*, 1992; Sorger, 1990) and ATF-2, an activator that requires an inducer to disrupt masking of the proline/Zn finger AD by interactions with the basic region-leucine zipper DBD (Li and Green, 1996). Alternate mRNA splicing can determine a transcription factor's function, as is the case for TFE3, a regulator of immunoglobulin gene promoters. The repressor TFE3S is a shorter isoform containing a C-terminal proline domain, and alternate splicing adds an N-terminal acidic domain which shows synergistic activation when combined with the proline region (Artandi *et al.*, 1995). Tissue specific splicing-in of a proline-rich exon

converts αNAC from a protein that associates with polypeptides exiting ribosomes to a DNA-binding activator of transcription in muscle cells (Yotov and St-Arnaud, 1996).

Activators and repressors share many general characteristics such as methods for promoter localization, amino acid composition of interaction domains, and targeting of multiple members of the PIC. As the list of known transcription factors and their mutations continues to grow, clues to the question of what activation (or repression) means biochemically are being uncovered through examinations of what steps in pol II transcription they influence.

Initiation

If activators increase the local concentration of a necessary factor at the promoter by recruitment, a simplistic test would be to artificially raise the overall concentration of that factor and thus bypass the need for recruitment. Such tests to determine whether TBP is limiting for pol II transcription *in vivo* have shown boosts in reporter activity after TBP plasmid transfections into monkey COS and *Drosophila* Schneider cells. In COS cells, a promoter dependent on the activating nuclear factor κB (NF-κB) was unaffected by additional TBP in the absence of the trans-activator, but cotransfection with NF-κB and TBP produced 20-fold greater transcription than with activator alone (Schmitz *et al.*, 1995). Several wild type *Drosophila* promoters, as well as a minimal TATA construct, were also enhanced by TBP over-expression, and the fold induction was inversely related to the promoter's normal strength (Colgan and Manley, 1992). Binding of TBP therefore appeared to be a rate-limiting step, a conclusion supported by experiments that produced activated transcription by tethering TBP to a promoter via fusion to specific DNA binding domains (Chatterjee and Struhl, 1995; Klages and Strubin, 1995; Xiao *et al.*, 1995).

Indirect recruitment of TBP through its TAFs also occurs. The *Drosophila* proteins Bicoid and Hunchback bind TAF110 and TAF60, respectively, and each alone can weakly promote DNA-TFIID association. When both are present, their binding affinity is synergistically increased and a multiplicative increase in transcription is observed (Sauer *et al.*, 1995; Sauer *et al.*, 1996).

A series of observations utilizing DNA footprinting revealed there is more to activation through TBP than its recruitment. Sequences adjacent to the TATA box showed extended protection in the presence of an activator (Horikoshi *et al.*, 1988), an effect now known to be due to the TAFs. Kinetic analyses with and without activators revealed that TBP-TAF binding to DNA and the extension of the footprint could be assayed sequentially, and that TBP mutants can be generated which are defective for activation but bind the activator, DNA, TFIIA, and TFIIB normally (Chi *et al.*, 1995; Hoopes *et al.*, 1992; Stargell and Struhl, 1996). Success with a technically difficult approach to measure activated *in vitro* transcription from the same DNA template population used for footprinting showed that with saturating concentrations of TFIID and TFIIA, the Epstein-Barr viral activator Zebra still enhanced transcription despite constitutive occupancy of the TATA box (Chi and Carey, 1996). The enhancement persisted even when the activator was removed by oligonucleotide competition before addition of the remaining PIC components, indicating that Zebra acts at the TFIID isomerization step. This isomerization, which follows TFIID-DNA binding but precedes entry of TFIIB, is apparently what produces the extended footprint and reflects stabilized TAF-DNA interaction and a change in conformation that favors PIC completion. Zebra

also has *in vitro* binding affinity for TFIIB, yet TFIIB rapidly entered the isomerized TFIID complex in the absence of activator.

Many other activator proteins have affinity for TFIIB. The investigation of whether binding events involving TFIIB are relevant to activated transcription started much the same as that for TBP. Over-expression of TFIIB in COS cells had no effect on basal or NF-κB activated promoter activity (Paal *et al.*, 1997; Schmitz *et al.*, 1995). In separate *Drosophila* experiments, TFIIB over-expression increased transcription only 1.1- or 2-fold for a Fushi tarazu (Ftz) activated reporter, and basal transcription in the absence of Ftz was either unaffected or increased 2-fold (Colgàn *et al.*, 1995; Colgan *et al.*, 1993). TFIIB would thus seem to not be limiting *in vivo*, reducing the potential need for its recruitment during activation. It has been noted that both TFIIB and TBP have charged regions carrying basic residues, and binding of activators to TFIIB may merely be the result of affinity for similar regions in the real target, TBP. As more activators are isolated, however, it has become apparent that there are classes with affinity for TFIIB only, both TBP and TFIIB, or TBP alone. Also, there are several domains within TFIIB involved in these interactions, some with characteristics quite different from TBP.

Studies with VP16, one of the first and strongest activators characterized, continue to play a large role in the question of TFIIB involvement during activated transcription. VP16 not only binds TBP and TFIIB *in vitro*, but also TBP-associated factor TAF32, TFIIH, and the co-activator PC4 (Ghosh *et al.*, 1996). TFIIB mutations that disrupt interaction with VP16 also prevent *in vivo* activation by VP16, but minimally affect basal transcription (Roberts *et al.*, 1993). An attempt to confirm these observations gave mixed results (Gupta *et al.*, 1996), and the promiscuous interactions of VP16 may

make it an overly complex activator for studying TFIIB specifically. Activation by the glutamine domain of Ftz can be blocked by co-expression with truncated TFIIB, and the degree of squelch is much greater than the effect on basal transcription (Colgàn *et al.*, 1995). Activation can be restored by introducing a mutation into the truncated TFIIB which blocks Ftz binding, or by adding wild-type TFIIB to compete the squelch. These and other squelch and rescue assays provide *in vivo* correlation of *in vitro* binding for activators with TFIIB, but questions remain regarding whether the TFIIB mutant truly does not affect basal PIC formation, and whether interaction with the over-expressed mutant might be artificially interfering with the actual target.

Progress toward understanding the role of TFIIB in activation will require additional mutation studies, for both activators and TFIIB, to attempt to specifically observe TFIIB related events. One interesting approach has been the creation of altered specificity mutations in TBP and TFIIB (Tansey and Herr, 1997). This system starts with a point mutant of TBP that does not bind TATA boxes, but instead recognizes a TGTAA binding site. A second TBP mutation in the TFIIB interaction domain swaps an arginine for a glutamic acid that normally forms an electrostatic contact with an arginine in TFIIB. Since this change places two basic residues in close proximity, the TBP-TFIIB complex does not form and transcription from a TGTAA promoter reporter is prevented. Activity is restored by a mutation of TFIIB, replacing the critical arginine with a glutamic acid. The serial altered specificity approach utilizes transcription that is independent of endogenous TBP and TFIIB, allowing mutant scans of both proteins, as well as activators, that can reveal differences in the ways TBP and TFIIB are targeted for activation.

Less progress has been made regarding other members of the PIC as regulatory targets for the initiation step. Protein interactions between regulators and pol II, TFIIF, TFIIE, TFIIH, and the mediator subunits have been observed. It is not known whether these represent redundant recruitment binding sites for the holoenzyme or if they have more specialized purposes. The largest subunit of TFIID, TAF250, has been shown to contain a protein kinase activity capable of autophosphorylation within each of two separate serine kinase domains. The combination of both domains in the context of the TFIID complex results in specific phosphorylation of the TFIIF component RAP74 (Dikstein *et al.*, 1996). Similar modifications or conformation changes of basal factors and pol II itself may be subject to regulation during transcription initiation. The relative importance of these mechanisms and those involving TBP and TFIIB probably depends on a number of factors including proximal promoter sequences, the types and number of upstream regulatory elements, and the host species. Yeast, for example, has TAF homologs that are apparently dispensable for transcription activation (Moqtaderi *et al.*, 1996; Walker *et al.*, 1996) and instead relies on the mediator fraction, while *Drosophila* and human systems are dependent upon TAFs for activation (Burley and Roeder, 1996).

Elongation

As described above, modification of the pol II CTD may be an important part of releasing the enzyme from the PIC for elongation. Other factors may assist with this release. Yeast SUB1, a homolog of the co-activator PC4, binds to TFIIB in a manner mutually exclusive to the TBP-TFIIB interaction and increased expression of SUB1 stimulates transcription *in vivo* (Knaus *et al.*, 1996). It was hypothesized that SUB1 acts

as a release factor capable of suppressing TFIIB mutations that prevent proper dissociation of the PIC for pol II elongation.

SUB1 and the HIV activator Tat primarily stimulate elongation, either through interactions at the PIC or with pol II directly, but other classes of activators can enhance both initiation and elongation. Multi-functional factors may concurrently or sequentially contact initial members of the PIC to promote complex formation and then induce conversion to elongation through interactions with other components such as TFIIH. Activators p53, E2F1, and VP16 can influence both steps, and they all bind several PIC subunits including TFIIH. A quadruple point mutant of VP16 has been described which no longer activates elongation but retains wild type activity for initiation enhancement (Blau *et al.*, 1996), and a different mutation suppresses the initiation effect but retains activity for elongation (Ghosh *et al.*, 1996). Other acidic ADs also activate initiation and elongation, but the effect on elongation requires more copies of the AD to be present at the promoter than are needed to boost initiation (Blair *et al.*, 1996).

Re-initiation

Some quantitative comparisons of basal and activated *in vitro* transcription indicate that activators do not generally increase the rate of PIC formation or pol II elongation, but increase the number of DNA templates occupied by PICs (Yamazaki *et al.*, 1990). In the case of the human activator Sp1, the overall equilibrium constant for PIC formation is also increased by 10-30 fold (Yean and Gralla, 1996). Since multiple rounds of transcription can occur *in vitro*, the resulting boost in RNA production is thus a function of not just more template copies being utilized but also enhanced levels of re-initiation, which is probably more relevant to activation of low copy number promoters *in*

vivo. Pol II escape from the PIC dissociates TFIIB and the other factors, but not TFIID, so the various activator interactions with these basal factors as described above may be more important for re-initiation than the initial PIC assembly.

A unique study utilizing a synthetic dimerizing ligand demonstrated the importance of re-initiation for promoter activation (Ho *et al.*, 1996). In yeast and human cells, two proteins were expressed, one containing an AD and the other a DBD, as fusions to a ligand binding domain. The cells were then incubated with the lipid-soluble dimeric ligand to link the DBD and AD, activating transcription from a reporter promoter. Addition of excess monomeric ligand quickly out-competed the dimer for ligand binding domains and prevented maintenance of the triple complex. Nuclear run-on analysis indicated that while the reconstituted activator directed initiation, its continued presence during re-initiation was necessary to produce RNA levels typical of activated transcription.

Before characterization of the PIC, research in transcriptional analysis concentrated on promoter sequences and the regulatory factors that bound those elements. One puzzle that became immediately apparent was the redundant use of DNA sequences acting as binding sites in promoters with different patterns of expression. Clearly not all specificity was at the level of what individual elements were recognized by particular factors, but also what combinations of elements were present and how the proteins that occupied them interacted. The complexity of the PIC now adds additional levels of specificity as transcription factors can target multiple patterns of individual proteins within the PIC as well as multiple steps during initiation and early elongation. TFIIB has received much attention as one of the central links between TFIID and pol II or

holoenzyme, and for its involvement in initiation (or re-initiation) and release for elongation. It will be interesting to see if the multiple roles of TFIIB represent another level at which promoter activity and specificity can be fine-tuned.

Transcription Factor IIB

X-ray crystallography and nuclear magnetic resonance spectroscopy have established partial structures for TFIIB. The C-terminal two-thirds of the sequence contains imperfect repeats, and in solution they form a bi-lobed structure separated by a cleft and joined by a short random coil linker (Bagby *et al.*, 1995). Each repeat contains five α-helices, and the C-terminus contains a short sixth α-helix (Fig. 1-1A). Structural instability in the N-terminal region of human TFIIB has prevented its inclusion in NMR and crystal structures, but the first 49 residues of a clone from a thermophilic Archaebacterium have been shown to form a Zn ribbon, which is the combination of a Zn finger and three anti-parallel β-sheets (Zhu *et al.*, 1996). Sequence homology among archaeal and eukaryotic TFIIB clones is high and structural conservation is expected.

The C-terminal repeats, termed the TFIIB core, have been co-crystallized with TBP bound to a TATA element (Nikolov *et al.*, 1995). TFIIB has numerous electrostatic interactions, hydrogen bonds, and van der Waals contacts with TBP and the DNA phosphodiester backbone (Fig. 1-1B), and is oriented with the C-terminus upstream of the TATA box and the Zn ribbon region downstream near the transcription start site (Leuther *et al.*, 1996). Other PIC proteins interact with TFIIB, and the interaction domains have been mapped by *in vitro* binding with TFIIB deletions and point mutations. RAP30 binding is disrupted by changes in the Zn region and helix A1, and can be displaced by

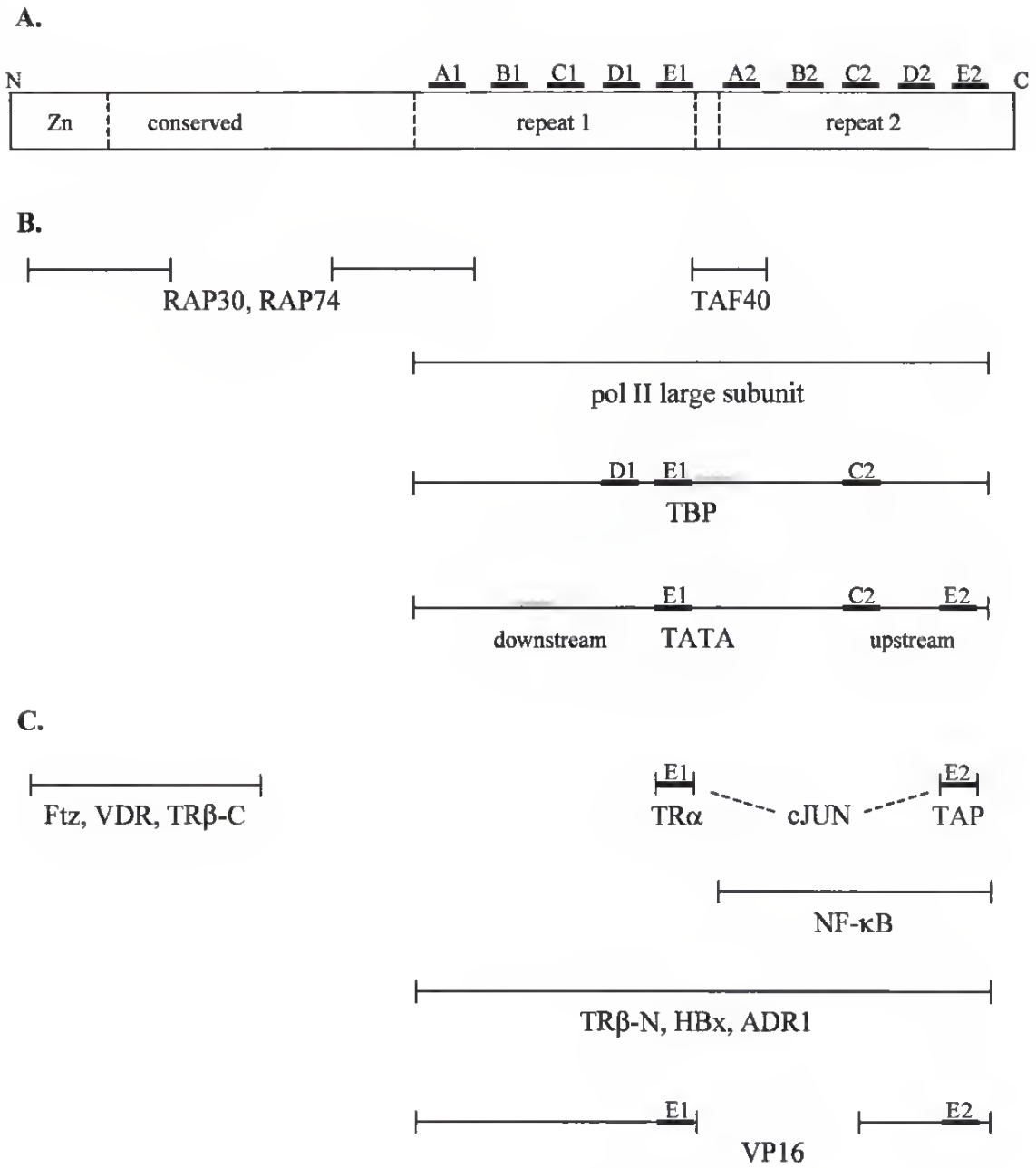


Figure 1-1. Transcription Factor IIB and its interaction domains.
A. General features of TFIIB include a Zn ribbon at the amino terminus, a region of high conservation, and two structural repeats each containing five alpha helices. **B.** TFIIB domains are shown that interact with components of the PIC. Clusters of residues important for binding are indicated with their helical label or, when between helices, as a dotted line. DNA and TBP contacts are from the crystal structure (Nikolov *et al.*, 1995) which utilized a truncated TFIIB; additional contacts are probably present in the N-terminal regions.
C. TFIIB domains that interact with various activators are found in the zinc ribbon and core repeats.

RAP74 (Fang and Burton, 1996). *Drosophila* TAF40 interacts with the linker-helix A2 region (Hori *et al.*, 1995) and pol II with the TFIIB core. An acidic domain near the CTD in the largest subunit of pol II seems to direct interactions with TFIIB, and those contacts can be competed by the acidic activation motifs of VP16 (Berroteran *et al.*, 1994; Xiao *et al.*, 1994).

Similar mapping techniques have been used with transcriptional activators, as shown in Fig. 1-1C. The Zn ribbon and adjacent conserved domain, which contains the sequences with highest homology among TFIIB clones, are the binding sites for Ftz, the vitamin D receptor (VDR), and thyroid hormone receptor β (TRβ-C terminal) (Baniahmad *et al.*, 1993; Colgàn *et al.*, 1995; Masuyama *et al.*, 1997). The core region binds a number of activators including c-Jun homodimers, the cellular co-activator TAP associated with HIV expression, NF-κB, hepatitis viral activator X (HBx), and the yeast activator of *Adh2* gene expression ADR1 (Chiang *et al.*, 1996; Franklin *et al.*, 1995; Lin *et al.*, 1997; Schmitz *et al.*, 1995; Yu *et al.*, 1995). VP16 interaction requires an intact first repeat of TFIIB and can be repressed by point mutations in helix E1 (Roberts *et al.*, 1993).

Roberts and Green (1994) also mapped an interesting intramolecular interaction in TFIIB. The conserved region binds to residues somewhere between the start of the linker and the end of repeat 2, and this interaction is disrupted by VP16. The resulting VP16-induced changes in TFIIB can be detected by altered patterns of protease cleavage products, leading to the suggestion that intra-peptide binding reduces TFIIB affinity for the PIC and a conformation change must occur for proper interaction and transcription initiation. This may explain the results of a recent study in yeast in which VP16

continued to activate despite a TBP mutation that prevented interaction with TFIIB (Lee and Struhl, 1997). VP16 may be acting to stabilize TFIID and catalyze the TFIIB conformation change, but does not enhance or even require TBP-TFIIB interaction to activate transcription. It would also seem to suggest that the presence of this activator can overcome some TBP point mutations that should severely disrupt PIC formation.

Other yeast TFIIB work has characterized additional functions. Two residues in the conserved domain that are identical among all TFIIB clones were identified as important for start site selection in a mutant screen (Pinto *et al.*, 1994). These glutamic acid and arginine residues are thought to form a salt bridge, because while individual mutations shifted the mRNA start site for several promoters, a charge swap partially restored the normal phenotype. The same mutants confer cold sensitivity that is suppressed by SUB1, and it was hypothesized that normal TFIIB release from TBP requires the ability to form the intra-peptide salt bridge (Knaus *et al.*, 1996). Human TFIIB will not functionally replace the yeast homolog *in vivo* unless helix B1 from the yeast clone is substituted for the same helix in the human protein (Shaw *et al.*, 1996). Four point mutations were generated to change residues within the yeast B1 helix to the human versions, and the resulting construct is functionally impaired, has a temperature-sensitive phenotype, and fails to respond to activators at some promoters (Shaw *et al.*, 1997). A screen for intragenic suppressors of this clone identified separate point mutants in helix C1 and at two residues in the conserved region that individually reversed the defect in transcriptional activation.

Increasingly detailed evidence continues to accumulate regarding the roles of TFIIB in both basal and activated transcription. Over 37 different transcriptional

regulators are now known to interact with TFIIB *in vitro*, and methodology continues to develop for precisely measuring their affinities for TFIIB and other PIC components and to relate *in vitro* binding to testable *in vivo* systems. Unfortunately, these biochemical and genetic models are being generated without input from a biologically and economically important kingdom, the plants. Research in plant transcriptional regulation has remained strong in promoter analysis and for characterization of signal transduction pathways that lead to transcriptional control, but the PIC targets of that regulation are lacking. When the work described in the following chapters was initiated, clones for TBP from *Arabidopsis*, maize, potato, and wheat had been isolated (Apsit *et al.*, 1993; Gasch *et al.*, 1990; Holdsworth *et al.*, 1992; Kawata *et al.*, 1992; Vogel *et al.*, 1993) as well as subunits of pol II from *Arabidopsis* and soybean (Dietrich *et al.*, 1990). It therefore seemed reasonable to next address the question of whether a TFIIB homolog exists in plants, and if so, what inferences could be made about plant pol II transcription by comparison of the factor with other eukaryotic homologs. Additionally, the availability of a number of plant transcriptional regulators makes rapid screening for TFIIB interactions possible and might provide new resources for investigating activation mechanisms.

CHAPTER 2
CLONING TFIIB HOMOLOGS FROM *ARABIDOPSIS* AND SOYBEAN

Literature Review

Protein sequencing of purified TFIIB and library screening using derived oligonucleotides allowed isolation of cDNA clones, first from baker's yeast (yTFIIB) (Pinto *et al.*, 1992) and then from human cell extracts (hTFIIB) (Ha *et al.*, 1991; Malik *et al.*, 1991). Sequence information suggested the presence of two regions with a repeated structural motif in the C-terminal two-thirds of the protein (core domain), and a possible Zn binding domain near the N-terminus. These features, as well as a highly conserved region adjacent to the Zn domain, are preserved in subsequent cDNA clones from *Drosophila* (Yamashita *et al.*, 1992), rat (Tsuboi *et al.*, 1992), *Xenopus* (Hisatake *et al.*, 1991), *Kluyveromyces* (Na and Hampsey, 1993), and two Archaebacteria (Creti *et al.*, 1993; Qureshi *et al.*, 1995).

When co-crystallized with *Arabidopsis* TBP and a consensus TATA box, hTFIIB contacts one stirrup of the saddle shaped TBP and also has clear interactions with the DNA phosphodiester backbone (Nikolov *et al.*, 1995). These interactions with TBP are expected to be similar for homologs from other species since the degree of amino acid conservation with hTFIIB is quite high, ranging from 79% identity with *Drosophila* to 94% and 99% identity among vertebrates (frog and rat). Yeast TFIIB, however, shows only 35% identity with the human version and contains 32 additional amino acids

compared to other eukaryotes. Archaeal clones are 33% identical to hTFIIB and have an extended N-terminal region.

Until now, no plant versions of TFIIB were available for comparison with other eukaryotic homologs. Plant TBP clones are around 83% identical to the corresponding region of human TBP, a remarkable degree of homology that is maintained in yeast and *Drosophila* (Hernandez, 1993). All archaeal and metazoan versions of TFIIB and TBP appear to be present as single copy genes, but there are two copies of TBP in *Arabidopsis*, maize and wheat, and protein sequences within each respective pair are 95%, 99% and 92% identical. This chapter describes the isolation and general characterization of cDNA clones for TFIIB from *Arabidopsis* and soybean. During analysis of these clones a partial cDNA with TFIIB homology was submitted to the EMBL database as part of an ongoing project to identify expressed sequence tags from *Arabidopsis* (Desprez *et al.*, 1994). This clone was kindly provided by the Arabidopsis Biological Resource Center at Ohio State University for sequence comparison.

Materials and Methods

cDNA Library Screening for Soybean and *Arabidopsis* TFIIB

Putative TFIIB cDNA clones were obtained by screening cDNA libraries with a synthetic oligomer derived from a highly conserved region of the TFIIB protein. To generate this probe, amino acid sequences for human, fruit fly, and yeast TFIIB were aligned using the CLUSTAL W analysis program (Thompson *et al.*, 1994), and a block of conserved residues near the end of repeat 1 was identified (amino acid residues 166 to 181, human). A 48 nucleotide oligomer was designed based on the DNA consensus

sequence for this conserved region and incorporated allowances for the soybean codon bias (Murray *et al.*, 1989). This oligomer, 5' ATTGCTTGCAGACAAGAAGGAGTT-CCAAGAACTTTCAAGGAAATTTGC 3', was ^{32}P end-labeled with T4 poly-nucleotide kinase to a specific activity of $3.2x10^8$ cpm/μg and used for hybridization screening with standard methods. Over 750,000 plaques from a *Glycine max* v. Resnik cDNA library in λgt11 (5'-Stretch, Clontech) were probed on duplicate nitrocellulose filter lifts in 1X SSC buffer (0.15 M NaCl, 0.015 M Na_3 citrate, pH 7.0) at 42° C. One clone gave a strong hybridization signal after two successive plaque purifications. The 800 bp insert DNA was PCR amplified with λgt11 primers using Vent polymerase (New England Biolabs) and subcloned to pUC118. Sequenase (DNA Sequencing Kit, version 2.0; United States Biochemical) dideoxy sequencing reactions were performed on a nested series of deletions created with DNA exonuclease III (Ausubel *et al.*, 1993), and all sequences reported are the result of at least two reactions on both top and bottom strand templates. Since this clone was an incomplete coding sequence, an internal *Pst*I/*Hin*dIII fragment was labeled by nick translation to re-probe $1.2x10^6$ plaques of the cDNA library. A second cDNA library prepared from 2,4-D treated soybean (*Glycine max* (L.) Merr. cv. Wayne) plumules in λgt10 was provided by Dr. Gretchen Hagen (Dietrich *et al.*, 1990), and the first round of screening 150,000 plaques with the internal fragment probe produced a single full length clone.

The soybean partial clone was also used to probe an *Arabidopsis thaliana* v. Colombia cDNA library in λgt11 (5'-Stretch, Clontech). After screening 660,000 plaques, eight were plaque purified twice and subcloned. The longest TFIIB isolate lacked sequences on both 5' and 3' ends but served as a probe for library re-screening of

300,000 plaques. Four additional clones were purified and ligated into pUC19, and the three longest were sequenced. Sequencing was also performed on the expressed sequence tag cloned into pBluescript SK-, ABRC DNA stock number ATTS3421.

Southern and Northern Blot Analysis of the *Arabidopsis* TFIIB Clone

The *Arabidopsis* TFIIB probe was generated from a pUC19 construct of the library isolate using PCR amplification directed by primers complementary to the 5' and 3' ends of the coding sequence. This 960 bp DNA was labeled by nick translation (Nick Translation System, Promega Corp.) to greater than $1x10^8$ cpm/μg and denatured by boiling for 10 min immediately before addition to the hybridization reactions.

Arabidopsis (Columbia) genomic DNA was isolated by the CTAB extraction method (Rogers and Bendich, 1994), and 10 μg were used for each restriction digestion containing 30 units each of *Xba*I or *Bgl*II individually, or *Bgl*II+*Eco*RI. After electrophoresis with markers in a 0.7% agarose gel and denaturation, the DNA was blotted to Hybond nylon membrane (Amersham) by downward capillary transfer (Turboblotter, Schleicher and Schuell) in 20X SSC buffer and then immobilized by UV crosslinking. Blots were prehybridized in 5 ml of APH buffer (5X SSC, 5X Denhardt's solution, 1% SDS, 100 μg/ml sheared denatured herring sperm DNA) for 15 min at 65° C (Ausubel *et al.*, 1993), followed by addition of probe to $1x10^6$ cpm/ml for overnight hybridization at the same temperature in a rotisserie incubation oven. Two 50 ml washes were performed at each of four stringency levels: 2X SSC/0.1% SDS at room temperature; and 0.2X SSC/0.1% SDS at room temperature, 42° C, and 60° C. Blots

were autoradiographed for three days at -70° C with amplification screens on Kodak XR-Blue X-ray film.

Arabidopsis total RNA was isolated by phenol extraction/LiCl precipitation (Pawlowski *et al.*, 1994) from four-week old whole plants grown at room temperature (control) or incubated at 37° C for two hours before extraction (heat shock). mRNA was purified using an oligo(dT) cellulose spin column (5'-3' Inc.) according to the manufacturer's instructions. Poly(A)$^+$ RNA (5 μg) from control and heat shock treatments was electrophoresed beside RNA molecular weight markers (0.24 - 9.4 kb, GibcoBRL) in a 1.0% agarose/formaldehyde gel and blotted as described above. Membranes were prehybridized in 5 ml FPH buffer (5X SSC, 5X Denhardt's solution, 50% formamide, 1% SDS, 100 μg/ml sheared denatured herring sperm DNA) for 15 min at 42° C, followed by addition of probe to 1x10^6 cpm/ml for overnight hybridization at 42° C. Washes at three stringency levels up to 42° C were performed as above, and the filters were autoradiographed overnight.

Primer Extension Mapping of *Arabidopsis* mRNA 5'-Terminus

Arabidopsis poly(A)$^+$ RNA (2 μg) was annealed to an oligomer of bottom strand sequence from 89 to 66 bp downstream from the first AUG: 5' ACCACACTCGGAGC-AAAGGGTATC 3'. The primer was ^{32}P end-labeled with T4 polynucleotide kinase and 0.3 μg was added to each reaction. Annealing in 2X reverse transcriptase buffer for two hours at 65° C was followed by slow cooling to room temperature. The reaction was diluted 1:2 with DTT and mixed dNTPs, and 400 units of SuperScript II reverse transcriptase (GibcoBRL) were added and incubated 2.5 hr at 42° C. The reaction was

stopped by adding loading buffer, heated to 65° C for 5 min, and electrophoresed on an 8.0% denaturing polyacrylamide gel. A dideoxy-adenosine sequencing reaction using the same primer and the pUC19-TFIIB plasmid as template was included as a size marker, and the gel was autoradiographed overnight.

Phylogeny Analysis of TFIIB cDNAs

DNA coding sequences for TFIIB from soybean, *Arabidopsis*, human, frog, fruit fly, yeast, and the thermophilic Archaebacterium *Pyrococcus woesei* were compared using the PAUP program (Swofford, 1991) after alignment by the CLUSTAL W algorithm. Bootstrap analysis was conducted in three separate trials with 100 repetitions each at the 90% confidence level, and trees were generated by midpoint rooting.

Results

After isolating a number of truncated soybean TFIIB clones, a 1226 bp sequence was recovered from the plumule cDNA library which seemed to be complete, but lacked nine codons at the 5' end compared to homologs from other species. Repeated attempts with various protocols for 5' RACE PCR amplification generated no clones containing additional coding or leader sequences. For comparison, a second plant TFIIB was cloned from *Arabidopsis* using the soybean TFIIB cDNA (*GmTFIIB*) as a screening probe. The largest *Arabidopsis* cDNA isolated was sequenced to reveal a 939 bp open reading frame. The predicted translations from both sequences produce 34.2 kDa proteins, and amino acid alignment shows 86% identity and 93% conservation of residues with functional similarity between *Arabidopsis* TFIIB1 (AtTFIIB1) and GmTFIIB. As indicated by

Figure 2-1. Comparison of TFIIB sequences from plants, metazoans, fungi and Archaebacteria.
Soybean (*G. max*) and *Arabidopsis* (*A. thaliana*) amino acid sequences for TFIIB were aligned with homologs from human, frog (*X. laevis*), fruit fly (*D. melanogaster*), yeast (*S. cerevisiae*), and the Archaebacterium *Pyrococcus woesei* by Clustal W analysis. Asterisks denote identical residues and dots conservative substitutions among the eukaryotic clones. Locations for α-helices A-E within the core repeats are indicated, as is the zinc ribbon domain and the four residues which coordinate Zn binding. Structural boundaries are according to Nikolov *et al.* (1995) and helix labels are from Bagby *et al.* (1995). The *P. woesei* isolate has an additional 23 amino acids at the N-terminus not shown.

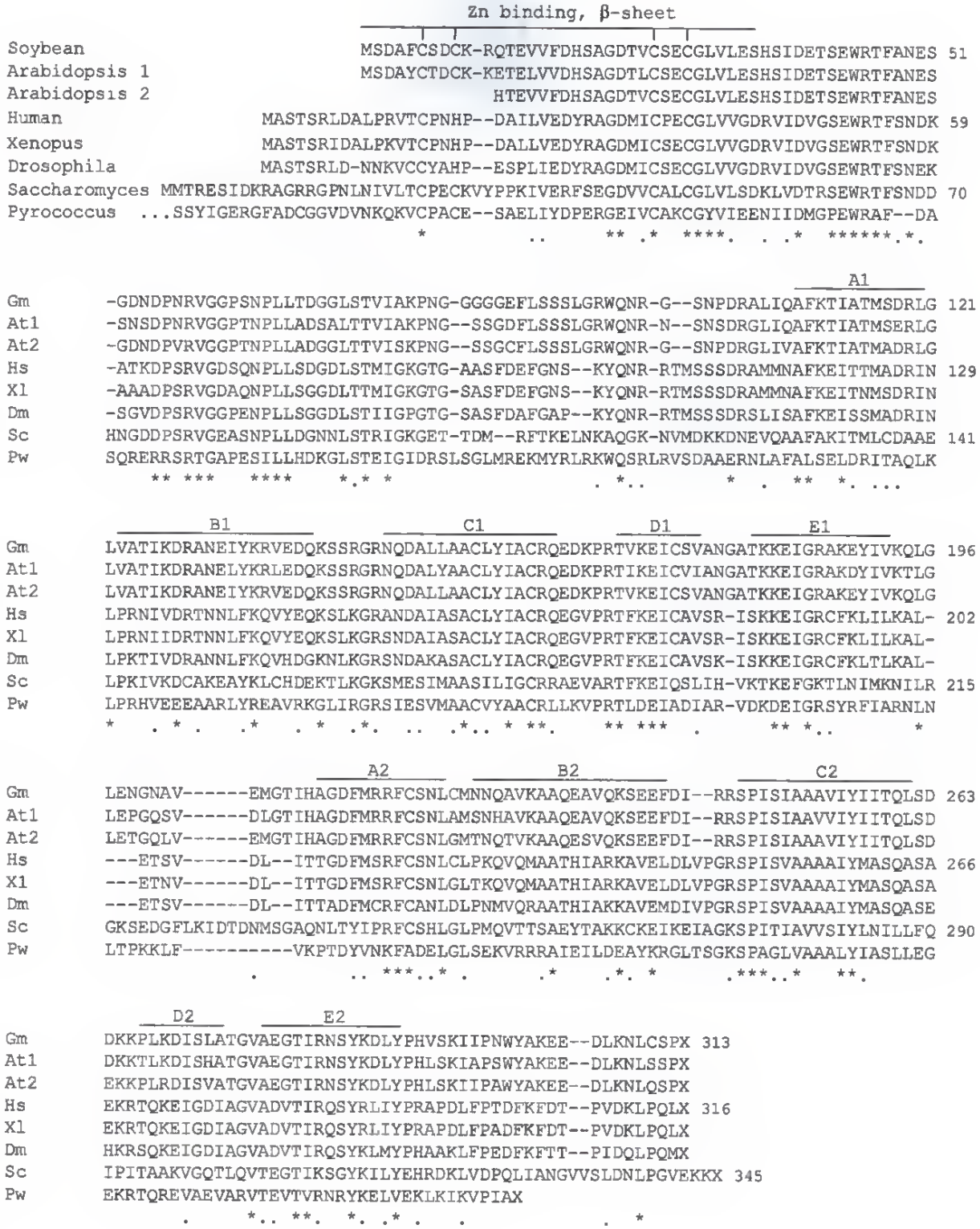

Zn binding, β-sheet
Soybean MSDAFCSDCK-RQTEVVFDHSAGDTVCSECGLVLESHSIDETSEWRTFANES 51
Arabidopsis 1 MSDAYCTDCK-KETELVVDHSAGDTLCSECGLVLESHSIDETSEWRTFANES
Arabidopsis 2 HTEVVFDHSAGDTVCSECGLVLESHSIDETSEWRTFANES
Human MASTSRLDALPRVTCPNHP--DAILVEDYRAGDMICPECGLVVGDRVIDVGSEWRTFSNDK 59
Xenopus MASTSRIDALPKVTCPNHP--DALLVEDYRAGDMICSECGLVVGDRVIDVGSEWRTFSNDK
Drosophila MASTSRLD-NNKVCCYAHP--ESPLIEDYRAGDMICSECGLVVGDRVIDVGSEWRTFSNEK
Saccharomyces MMTRESIDKRAGRRGPNLNIVLTCPECKVYPPKIVERFSEGDVVCALCGLVLSDKLVDTRSEWRTFSNDD 70
Pyrococcus ...SSYIGERGFADCGGVDVNKQKVCPACE--SAELIYDPERGEIVCAKCGYVIEENIIDMGPEWRAF--DA
A1
Gm -GDNDPNRVGGPSNPLLTDGGLSTVIAKPNG-GGGGEFLSSSLGRWQNR-G--SNPDRALIQAFKTIATMSDRLG 121
At1 -SNSDPNRVGGPTNPLLADSALTTVIAKPNG--SSGDFLSSSLGRWQNR-N--SNSDRGLIQAFKTIATMSERLG
At2 -GDNDPVRVGGPTNPLLADGGLTTVISKPNG--SSGCFLSSSLGRWQNR-G--SNPDRGLIVAFKTIATMADRLG
Hs -ATKDPSRVGDSQNPLLSDGDLSTMIGKGTG-AASFDEFGNS--KYQNR-RTMSSSDRAMMNAFKEITTMADRIN 129
Xl -AAADPSRVGDAQNPLLSGGDLTTMIGKGTG-SASFDEFGNS--KYQNR-RTMSSSDRAMMNAFKEITNMSDRIN
Dm -SGVDPSRVGGPENPLLSGGDLSTIIGPGTG-SASFDAFGAP--KYQNR-RTMSSSDRSLISAFKEISSMADRIN
Sc HNGDDPSRVGEASNPLLDGNNLSTRIGKGET-TDM--RFTKELNKAQGK-NVMDKKDNEVQAAFAKITMLCDAAE 141
Pw SQRERRSRTGAPESILLHDKGLSTEIGIDRSLSGLMREKMYRLRKWQSRLRVSDAAERNLAFALSELDRITAQLK
B1 C1 D1 E1
Gm LVATIKDRANEIYKRVEDQKSSRGRNQDALLAACLYIACRQEDKPRTVKEICSVANGATKKEIGRAKEYIVKQLG 196
At1 LVATIKDRANELYKRLEDQKSSRGRNQDALYAACLYIACRQEDKPRTIKEICVIANGATKKEIGRAKDYIVKTLG
At2 LVATIKDRANEIYKRVEDQKSSRGRNQDALLAACLYIACRQEDKPRTVKEICSVANGATKKEIGRAKEYIVKQLG
Hs LPRNIVDRTNNLFKQVYEQKSLKGRANDAIASACLYIACRQEGVPRTFKEICAVSR-ISKKEIGRCFKLILKAL- 202
Xl LPRNIIDRTNNLFKQVYEQKSLKGRSNDAIASACLYIACRQEGVPRTFKEICAVSR-ISKKEIGRCFKLILKAL-
Dm LPKTIVDRANNLFKQVHDGKNLKGRSNDAKASACLYIACRQEGVPRTFKEICAVSK-ISKKEIGRCFKLTLKAL-
Sc LPKIVKDCAKEAYKLCHDEKTLKGKSMESIMAASILIGCRRAEVARTFKEIQSLIH-VKTKEFGKTLNIMKNILR 215
Pw LPRHVEEEAARLYREAVRKGLIRGRSIESVMAACVYAACRLLKVPRTLDEIADIAR-VDKDEIGRSYRFIARNLN
A2 B2 C2
Gm LENGNAV------EMGTIHAGDFMRRFCSNLCMNNQAVKAAQEAVQKSEEFDI--RRSPISIAAAVIYIITQLSD 263
At1 LEPGQSV------DLGTIHAGDFMRRFCSNLAMSNHAVKAAQEAVQKSEEFDI--RRSPISIAAVVIYIITQLSD
At2 LETGQLV------EMGTIHAGDFMRRFCSNLGMTNQTVKAAQESVQKSEEFDI--RRSPISIAAAVIYIITQLSD
Hs ---ETSV------DL--ITTGDFMSRFCSNLCLPKQVQMAATHIARKAVELDLVPGRSPISVAAAAIYMASQASA 266
X1 ---ETNV------DL--ITTGDFMSRFCSNLGLTKQVQMAATHIARKAVELDLVPGRSPISVAAAAIYMASQASA
Dm ---ETSV------DL--ITTADFMCRFCANLDLPNMVQRAATHIAKKAVEMDIVPGRSPISVAAAAIYMASQASE
Sc GKSEDGFLKIDTDNMSGAQNLTYIPRFCSHLGLPMQVTTSAEYTAKKCKEIKEIAGKSPITIAVVSIYLNILLFQ 290
Pw LTPKKLF----------VKPTDYVNKFADELGLSEKVRRRAIEILDEAYKRGLTSGKSPAGLVAAALYIASLLEG
D2 E2
Gm DKKPLKDISLATGVAEGTIRNSYKDLYPHVSKIIPNWYAKEE--DLKNLCSPX 313
At1 DKKTLKDISHATGVAEGTIRNSYKDLYPHLSKIAPSWYAKEE--DLKNLSSPX
At2 EKKPLRDISVATGVAEGTIRNSYKDLYPHLSKIIPAWYAKEE--DLKNLQSPX
Hs EKRTQKEIGDIAGVADVTIRQSYRLIYPRAPDLFPTDFKFDT--PVDKLPQLX 316
Xl EKRTQKEIGDIAGVADVTIRQSYRLIYPRAPDLFPADFKFDT--PVDKLPQLX
Dm HKRSQKEIGDIAGVADVTIRQSYKLMYPHAAKLFPEDFKFTT--PIDQLPQMX
Sc IPITAAKVGQTLQVTEGTIKSGYKILYEHRDKLVDPQLIANGVVSLDNLPGVEKKX 345
Pw EKRTQREVAEVARVTEVTVRNRYKELVEKLKIKVPIAX

Figure 2-2. DNA homology of plant clones for TFIIB.
Alignment of coding regions from cDNAs for *AtTFIIB1* (top) and *GmTFIIB* (bottom) indicates 77% conservation.

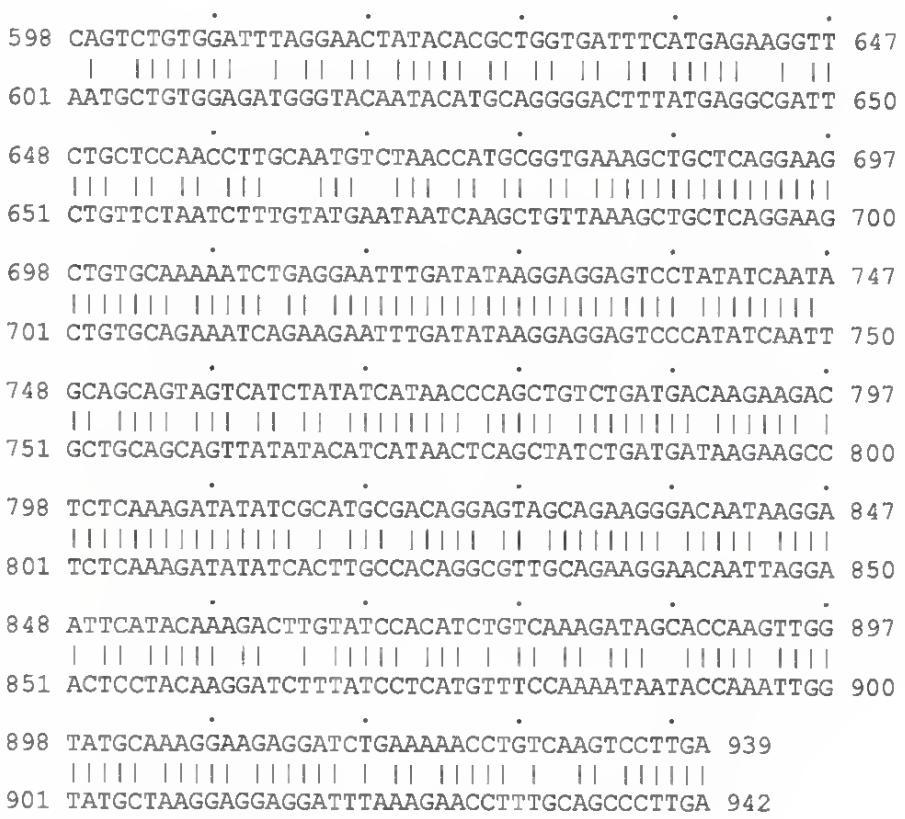


Figure 2-2 -- continued

CLUSTAL alignment (Fig. 2-1), both plant factors show extensive homology to versions of the protein found in other eukaryotes and in Archaebacteria. AtTFIIB1 is about 46% identical and 62% conserved to the human, fruit fly, and frog homologs; and is 33% identical and 54% conserved to yeast. DNA sequences for the coding regions of both plant genes are 77% identical (Fig. 2-2) and are available at GenBank accession numbers U31096 (*AtTFIIB1*) and U31097 (*GmTFIIB*). Subsequent sequencing of the expressed sequence tag from *Arabidopsis* indicates a second gene copy, *AtTFIIB2*, may be present for this protein. The isolate lacks 11 codons at the 5' end compared to *AtTFIIB1*, and the remaining open reading frame encodes a protein 87% identical, 93% conserved to AtTFIIB1 (Fig. 2-1).

The cDNA for *GmTFIIB* contains only 50 bp of sequences upstream from the first ATG, and since the reading frame remains open in this region, the possibility of additional codons could not be ruled out. Analysis of the *AtTFIIB1* cDNA, however, suggests that plant TFIIB genes are indeed slightly shorter at the amino terminus because the first ATG codon in the open reading frame of *AtTFIIB1* aligns with the putative start codon in *GmTFIIB*. In contrast to *GmTFIIB*, the predicted leader sequence of *AtTFIIB1* contains a stop codon 29 bp upstream from the first methionine which serves to further delimit the reading frame. It was unclear from the sequence analysis if this stop codon was a part of the *AtTFIIB1* untranslated leader since the first isolate for *AtTFIIB1* carries an artifact from library construction. The resulting clone is a fusion with the 60S ribosomal protein L29 such that the 5' leader regions are joined and the open reading frames are in opposite orientations. To measure whether the true 5' untranslated sequence

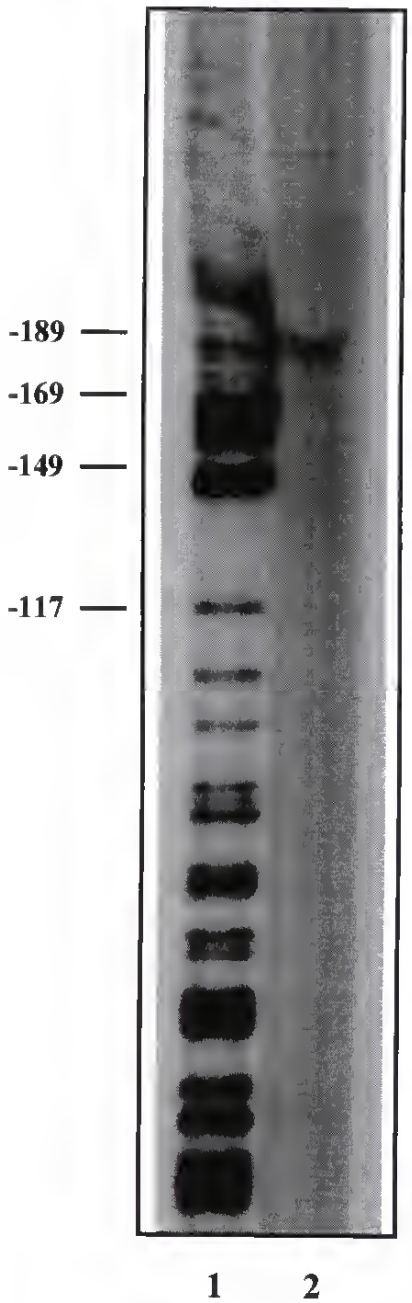


Figure 2-3. Primer extension of AtTFIIB mRNA.
Sequence markers were produced from the pUC19-*AtTFIIB1* plasmid using a bottom strand primer, and positions upstream from the first ATG are indicated (lane 1). Reverse transcription (lane 2) from the same primer annealed to *Arabidopsis* mRNA produced a cDNA with an endpoint approximately 180 nucleotides upstream from the first AUG.

of *AtTFIIB1* is long enough to include the observed stop codon, a primer extension reaction was conducted using *Arabidopsis* mRNA. The 5' terminus of the *AtTFIIB1* mRNA was shown to be 180 nucleotides upstream of the putative start codon (Fig. 2-3), indicating that the cDNA may actually contain the complete N-terminus of the protein since the mRNA leader is more than long enough to include the in-frame stop codon. Concurrent with this experiment, two additional independent clones for *AtTFIIB1* were recovered from the library and sequenced; neither contained the artificial fusion and both confirmed the sequence of the 5' untranslated leader including the stop codon.

Genomic blots were probed with the coding region of the *AtTFIIB1* cDNA in order to obtain an estimate of copy number and to confirm the plant origin of the clone. The cDNA contains one *Bgl*II restriction site in the 5' leader sequence and no internal *Xba*I sites. A single band hybridizing at high stringency to the cDNA probe was observed when genomic DNA was digested with *Xba*I (Fig. 2-4A). Two bands resulted from *Bgl*II digestion, one of which is shorter than the cDNA and probably resulted from the presence of a second site within an intron. An *Eco*RI restriction site is present in the coding region 90 bp upstream from the stop codon. Double digests with *Eco*RI and *Bgl*II are predicted to produce a 920 bp fragment if no introns are present. Since the genomic fragments detected were approximately 1600 bp and 550 bp in size, a minimum estimate for intron sequences in *AtTFIIB1* would be 1230 bp. Moderate stringency washing revealed additional hybridizing bands which may be the result of *AtTFIIB2*, or other sequences with homology to TFIIB such as TFIIIB (Fig. 2-4B).

Northern blots were conducted to demonstrate that the *AtTFIIB1* gene is expressed, determine the size of the *AtTFIIB1* transcript, and to complement ongoing

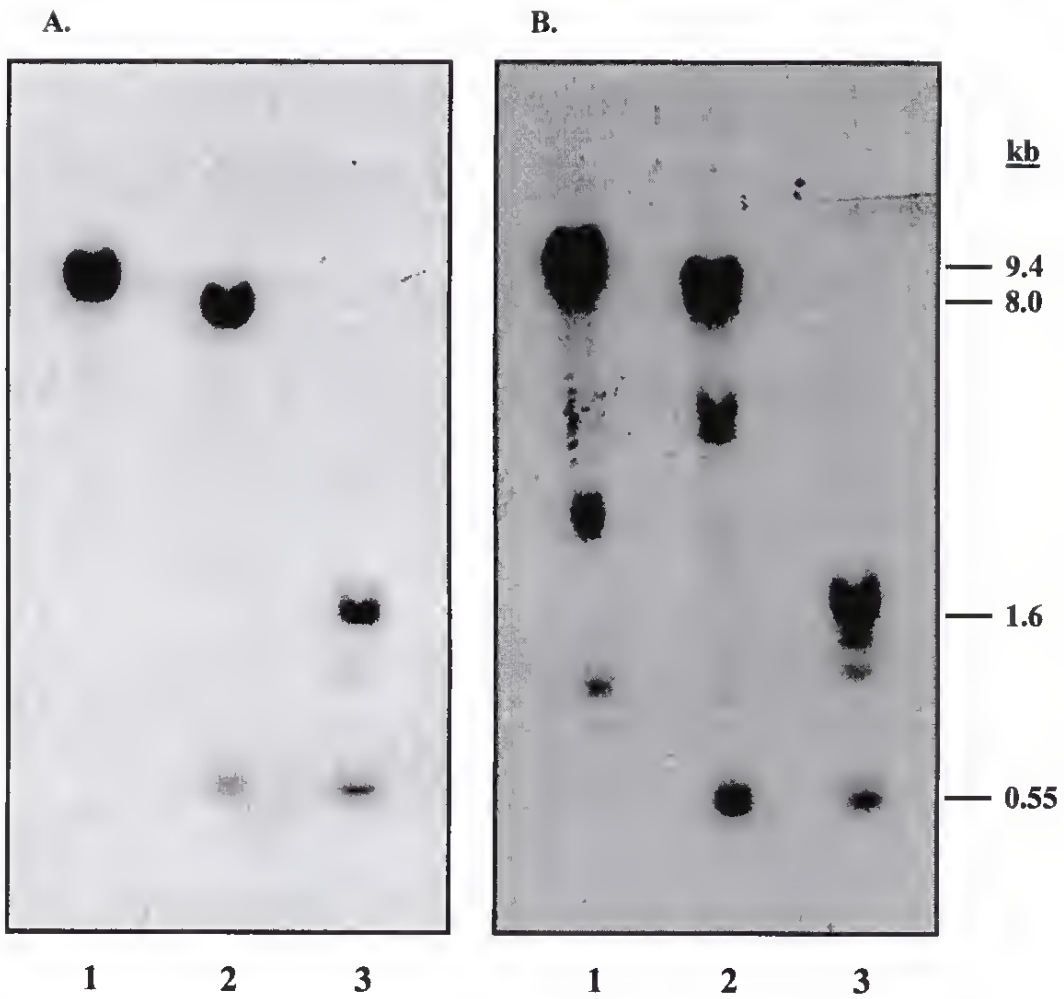


Figure 2-4. Southern analysis of *AtTFIIB1*.
A. *Arabidopsis* genomic DNA (5 μg per reaction) was restriction digested with *Xba*I (lane 1), *Bgl*II (lane 2), or *Eco*RI + *Bgl*II (lane 4), blotted and probed with the coding region of *AtTFIIB1* cDNA, and washed at 60° C. **B.** Genomic DNA (10 μg) was digested and probed as in (A.), but blots were washed at 42° C. Fragment length estimates in kilobase pairs are indicated.

Figure 2-5. *AtTFIIB1* Northern analysis.
Arabidopsis poly(A)$^+$ RNA (5 µg per lane) was blotted and then probed with the coding region of *AtTFIIB1* cDNA. The mRNA was isolated from control (lane 1) and heat-shocked (lane 2) whole plants.

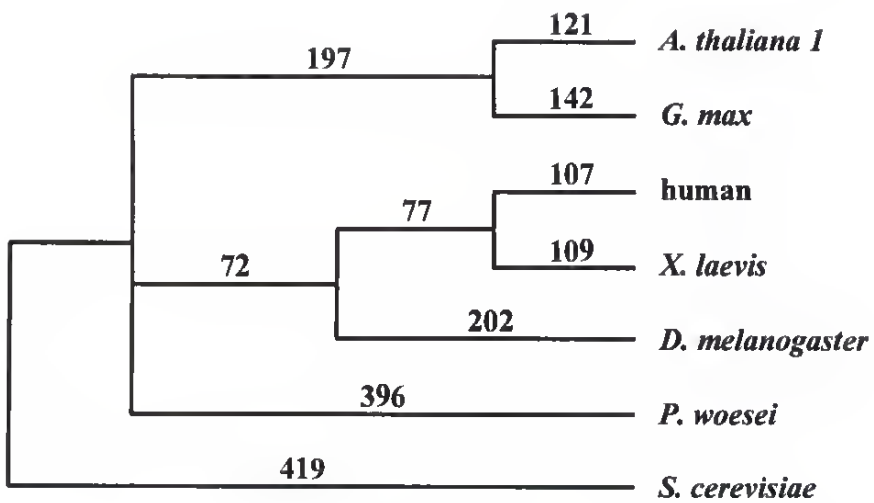


Figure 2-6. TFIIB phylogram.
DNA sequences from the coding regions of clones from Figure 1 were compared by parsimony analysis. Length units indicate the number of nucleotide changes between shared branch points.

studies related to heat stress in plants. Equal amounts of *Arabidopsis* poly(A)$^+$ RNA from control and heat shocked plants were analyzed (Fig. 2-5). A transcript of approximately 1400 nucleotides was detected, with no apparent change in abundance after heat stress. After the hybridization reaction, the membrane was washed at high stringency for RNA blots (0.2X SSC/0.1% SDS, 42° C) and similar high stringency washes for the Southern blots showed no cross-hybridization between *AtTFIIB1* and *AtTFIIB2* with this probe. Confirmation of the *AtTFIIB1* expression pattern and comparison to the mRNA length of *AtTFIIB2* will require additional Northern blots using specific probes derived from the untranslated cDNA regions of each clone.

Discussion

Plant versions of TFIIB retain the overall structural organization observed among animal, fungal, and Archaebacterial homologs previously characterized. The zinc binding domain, adjacent conserved region, and core repeats show similar lengths and organization within the protein, no doubt reflecting an ancient common origin for the basal transcription mechanism (Ouzounis and Sander, 1992). Four cysteines, or three cysteines and one histidine, coordinate metal ion binding in the Zn ribbon, and the adjacent conserved regions show several blocks of amino acid identity. A portion of this conserved region in hTFIIB was postulated to form an intramolecular association with the second repeat, perhaps folding the protein into a conformation that is altered upon binding with acidic activators (Roberts and Green, 1994). Whether this also occurs in plants is to be determined. Mutations in this domain alter the mRNA start site in yeast (Pinto *et al.*, 1994), and the two residues affected by those mutations are identical in the

three plant clones and in all other homologs. Another functional domain characterized in yeast is helix B1, which when swapped between yeast and human homologs confers hTFIIB with the ability to support yeast cell growth (Shaw *et al.*, 1996). Plant sequences for this helix are more like those of human than yeast, with eight of 16 residues conserved with yTFIIB and 12 conserved with hTFIIB.

Phylogenetic analysis confirmed previous reports (Ouzounis and Sander, 1992; Qureshi *et al.*, 1995) that TFIIB homologs sort into equidistant groups corresponding to taxonomic kingdoms. Our parsimony tree places human, *Xenopus*, and *Drosophila* clones in one branch (metazoan) and plants, yeast, and Archaebacteria on three separate branches (Fig. 2-6). It is interesting to note that the number of differences detected between two dicots, *Arabidopsis* and soybean, is nearly the same as that between vertebrates and insects. *AtTFIIB2* was not included in the tree because its coding region is incomplete, but a pair-wise comparison with the partial cDNA sequence indicated it is 76% homologous to *AtTFIIB1,* and *AtTFIIB1* shows 77% homology with *GmTFIIB.*

The two full-length TFIIB proteins from plants show a large degree of similarity in amino acid sequences throughout the length of the protein. This pattern is also characteristic among other species when TFIIB comparisons are made within the same kingdom. In comparisons between kingdoms, regions of conservation emerge which are most evident in three areas: blocks of identity within the 47 amino acids C-terminal to the Zn domain (residues 30 to 76 of plant TFIIBs), and the two repeat motifs. One of the regions of variability between kingdoms is the linker between the two core repeats. The linker is a random coil (Bagby *et al.*, 1995) and varies in length from 17 amino acids in human, rat, frog and fly to 23 in plants and 29 in yeast. In the Archaebacterium

Sulfolobus shibatae, the linker is from 9 to 18 residues in length depending on the location of the core repeat boundaries (Qureshi *et al.*, 1995). It is not known if the presence of additional residues in the plant linker, compared to hTFIIB, affects the conformation or distance between repeats, or merely reflects further looping out of the polypeptide chain. It would seem, however, that binding interactions with TBP and DNA tolerate differences in linker length since the ternary co-crystal structure was successfully resolved using *Arabidopsis* TBP (AtTBP) and hTFIIB (Nikolov *et al.*, 1995). Close interactions were detected between the two proteins at D207 and L208 in the hTFIIB linker. These residues are identical in AtTFIIB1 and show conservative substitution in GmTFIIB when aligned by the CLUSTAL analysis.

Nikolov *et al.* (1995) identified a number of van der Waals contacts, salt bridges, and hydrogen bonds in the DNA-AtTBP-hTFIIB interaction. Of the 27 hTFIIB residues involved in these contacts, 21 are identical and three conserved in the AtTFIIB1 sequence and 19 are identical, five conserved in GmTFIIB. The three variant amino acid residues occur near helix C2 and within helix E1. hTFIIB residue G247 near helix C2 contacts the DNA backbone, and in plants this position is occupied by arginine, suggesting that a charge interaction with the DNA may occur.

The other two points of variance between plant and metazoan proteins occur in helix E1, which has been shown to have several roles in the function of TFIIB, including binding with activators and TBP. A helical wheel projection of this domain (Fig 2-7A) indicates that the three residues that interact with the C-terminal stirrup of AtTBP (hTFIIB K188, G192, and F195) cluster to one face along one-fourth of the helical surface. Both plant clones have substituted F195 with lysine, changing the nature of this

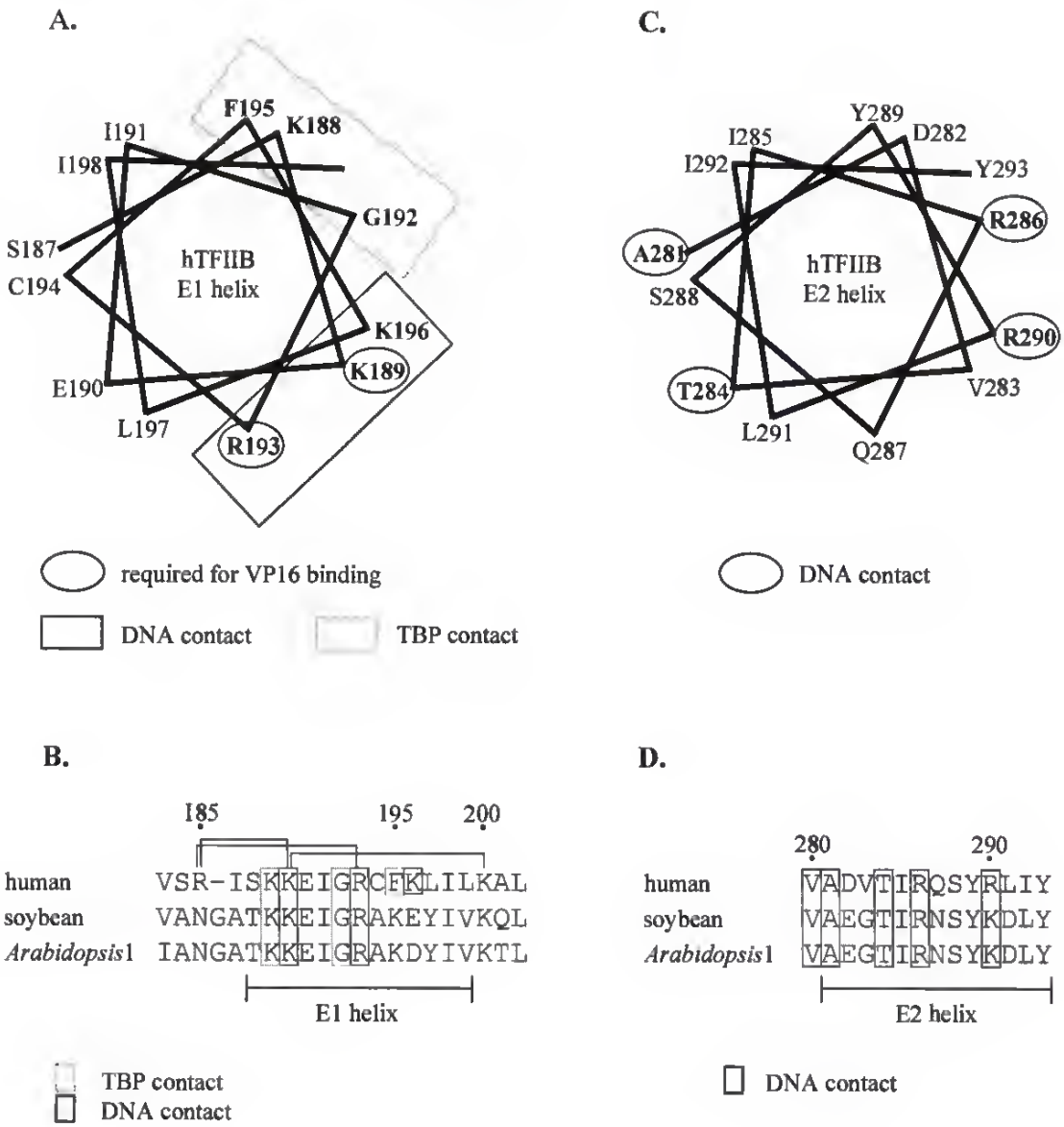


Figure 2-7. Interactions with and comparisons between the E1 and E2 α-helices of TFIIB. **A.** A helical wheel representation of the amphipathic E1 helix of hTFIIB shows sites of contact with TBP and the DNA backbone occur on two adjacent faces. **B.** hTFIIB mutants (residue pairs joined by brackets) that disrupt VP16 binding occur at sites that overlap DNA contact points within and flanking the E1 helix (Roberts *et al.*, 1993). Four of the six residues interacting with DNA or TBP are conserved between human and plant clones. **C.** The helical wheel projection for hTFIIB helix E2 shows DNA contact sites map to opposite faces. **D.** DNA-binding residues in the E2 helix region are conserved. Contact points with TBP and DNA were determined for hTFIIB by Nikolov *et al.* (1995).

position from hydrophobic non-polar to basic. While this hTFIIB residue forms a van der Waals contact with a glutamic acid in TBP, the corresponding plant interaction is perhaps electrostatic instead. Three more residues contact the DNA backbone (hTFIIB K189, R193, and K196), and occupy a second face of the helix. Double mutation of two of these, K189 and R193, disrupts binding of the acidic activator VP16 (Roberts *et al.*, 1993), which indicates a somewhat unusual overlap of functions on the same helical face. In plants, K196 is changed to a glutamic acid (GmTFIIB) or aspartic acid (AtTFIIB1) (Fig. 2-7B), altering the charge from basic to acidic and decreasing the likelihood of interaction at this residue with either DNA or an acidic activator domain. In both yeast versions, this position is occupied by polar uncharged amino acids. Since acidic activators function in both plants and yeast, the two invariant basic residues along the E1 helical face must be sufficient for activation of transcription, in combination with other sites of interaction that may utilize not just charge but also the bulky hydrophobic residues often observed within acidic domains. VP16, for example, also binds hTFIIB helix E2, the analog to E1 in the second repeat which has no interaction with TBP but five contacts with DNA. Two of these residues are basic and are located on one helical face, while the remaining three are uncharged and on the opposite side, a pattern that is identical in the plant clones (Fig. 2-7C and D). This opposite face contains central hydrophilic residues surrounded by hydrophobic groups, some of which may facilitate VP16 binding via the hydrophobic amino acids flanking its acidic domains. Functional conservation of this TFIIB region appears high since eight of nine other residues also within helix E2, but not in contact with DNA, are identical or conserved between metazoans and plants.

CHAPTER 3
TATA-BINDING PROTEIN AFFINITY AND SPECIES SPECIFICITY OF *ARABIDOPSIS* TRANSCRIPTION FACTOR IIB

Literature Review

Newly cloned homologs of TFIIB are often functionally characterized by testing their ability to interact with a DNA-TBP complex and to support transcription in a reaction lacking endogenous TFIIB. Interaction with DNA-TBP has generally been measured by the electrophoresis mobility shift assay (EMSA) (Ha *et al.*, 1993), in which a radiolabeled DNA probe is allowed to interact in solution with the binding protein(s) and the complex is electrophoresed in a native polyacrylamide gel. Probe interacting with protein will exhibit slower mobility compared to free DNA, and binding is stabilized upon exposure to the electric field by caging effects of the polyacrylamide matrix (Fried and Crothers, 1981; Garner and Revzin, 1981). EMSAs for the DNA-TBP and DNA-TBP-TFIIB interactions have given variable results, depending on the EMSA conditions and the source and purity of the TBP protein.

Initial binding studies with the yeast TFIID fraction required TFIIA to produce a stable interaction (Buratowski *et al.*, 1989). The subsequent use of recombinant yeast TBP (yTBP) allowed its characterization in the absence of TAFs or other TFIID proteins, and demonstrated TATA interaction without a requirement for TFIIA (Horikoshi *et al.*, 1989; Kao *et al.*, 1990; Peterson *et al.*, 1990). Recombinant human TBP (hTBP) directs

formation of a more stable complex with TFIIA and TFIIB than does yTBP (Maldonado *et al.*, 1990). Under conditions optimized for TFIIB binding, there is sometimes no DNA interaction with yTBP alone (Buratowski and Zhou, 1993), and hTBP often forms a DNA-TFIIB triple complex that does not resolve as a separate band but instead is an extended region of enhanced probe binding (Thompson *et al.*, 1995; Yamashita *et al.*, 1993). The presence of TFIIB in these cases can be confirmed by addition of antibodies raised against TFIIB, producing a supershifted signal present only when TFIIB has interacted with the labeled probe through TBP (Maldonado *et al.*, 1990; Thompson *et al.*, 1995). EMSAs have been used to estimate the apparent equilibrium dissociation constant (K_d) of yTBP bound to a TATA probe as 2 nM and the K_d of potato TBP as 5 nM, and the rates of association and dissociation were described as slow (Hahn *et al.*, 1989; Holdsworth *et al.*, 1992).

The EMSA technique has been combined with DNase I protection assays to more accurately measure TBP binding kinetics. DNA binding proteins block access to the sequences they bind, preventing DNase I digestion in that region and generating a footprint that can be resolved by electrophoresis on sequencing gels. Several reactions can be run in parallel to titrate the amount of protein required to occupy a binding element, and this approach was used to estimate the yTBP K_d as 3 nM (Hoopes *et al.*, 1992). It was also observed that the rate of dissociation (k_d) remained slow but the rate of association (k_a) was quite rapid. The differences in k_a between this and previous experiments were attributed to the use of higher TBP concentrations. DNase I footprinting of yTBP showed a 2.5-fold increase in TATA affinity when TFIIB was added and no effect with the addition of TFIIA (Imbalzano *et al.*, 1994). Altering the binding

buffer to create sub-optimal conditions for yTBP, however, produced a 10-fold increase in binding affinity when either hTFIIB or yeast TFIIA was included. No estimates for the K_d of TFIIB or TFIIA binding to a DNA-TBP complex have been reported.

An alternative assay for protein-DNA binding utilizes fluorescence anisotropy (Lundblad *et al.*, 1996). An oligonucleotide labeled with a fluorescein tag is exposed to polarized light at the excitation wavelength, and if no molecular motion occurs all the resulting fluorescence emission will remain polarized and is detected in the original plane. In solution, however, the DNA rapidly rotates, carrying the excited tag out of the original plane of polarization before emission and reducing the amount of detected fluorescence. Since the rate of rotation is related to the molecular volume, interaction between the oligomer and a DNA-binding protein will result in a complex with increased volume, slower rotation, and less signal leaving the plane of excitation (Cantor and Schimmel, 1980). In practice, anisotropy (A) is measured by detecting fluorescence intensities (I) in both the excitation plane ($I_{||}$, polarizing filter for detection set parallel to excitation filter) and perpendicular to that plane ($I_{\perp}$). The result of calculating

$$A = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}}$$

expresses the rotational depolarization of the measured fluorescence; the denominator indicates total emission intensity and the mathematical maximum for A is 0.4 (no molecular rotation) (Cantor and Schimmel, 1980; Jameson and Sawyer, 1995). Several DNA-transcription factor complexes have been analyzed by fluorescence anisotropy, including the binding of CREB to its cAMP-responsive enhancer element and the

subsequent association of a co-activator, CBP (Lundblad *et al.*, 1996). Advantages of this method compared to standard EMSAs include having all reactants at equilibrium in solution rather than in a gel matrix and being able to rapidly measure multiple interaction reactions in real time under various buffer conditions.

A second functional test for TFIIB is the ability to support *in vitro* transcription. Multi-column chromatography protocols are used to fractionate nuclear extracts, and when the fractions are appropriately re-mixed transcription can be detected, often using a specialized template or primer extension to ensure the assay measures RNA specifically initiated from the pol II promoter. A functional recombinant TFIIB protein should substitute for fraction B after *E. coli* expression and purification (Tsuboi *et al.*, 1992; Wampler and Kadonaga, 1992; Yamashita *et al.*, 1992). Similar experiments can be performed on crude nuclear extracts that have been specifically depleted for the factor of interest using immobilized antibodies or, as was the case for hTFIIB, a column of immobilized VP16 activation domain (Roberts *et al.*, 1993).

Besides a few activation studies in monkey and *Drosophila* cells (Colgàn *et al.*, 1995; Paal *et al.*, 1997; Schmitz *et al.*, 1995), most *in vivo* characterizations of TFIIB have been in yeast. Mutations affecting the transcription start site have been mapped to the conserved region of yTFIIB adjacent to the Zn domain (Pinto *et al.*, 1994), and suppressors of these mutants have been used to identify interactions with pol II (Berroteran *et al.*, 1994; Sun *et al.*, 1996; Xiao *et al.*, 1994) and other cellular factors (Sun and Hampsey, 1996). Yeast plasmid shuffle experiments in which chromosome-encoded yTFIIB is replaced with a plasmid-borne copy have detected differences between the *K. lactis* and *S. cerevisiae* homologs (Na and Hampsey, 1993) and indicate that rat

and hTFIIB are unable to substitute for yTFIIB (Shaw *et al.*, 1996; Tschochner *et al.*, 1992). Promoter-specific differences have also been observed with *in vitro* comparisons of *Drosophila* and hTFIIB (Wampler and Kadonaga, 1992).

A series of yeast/human TFIIB hybrids was constructed to map the domain responsible for incompatibility *in vivo*, and species specificity was localized to helix B1, the second α-helix in TFIIB repeat 1 (Shaw *et al.*, 1996). Gain-of-function experiments with an inactive yTFIIB carrying a substituted human B1 helix confirmed that four residues within the helix are critical for proper function; their reversal to the yeast versions restored activity (Shaw *et al.*, 1997). Point mutations were generated to change yTFIIB to the hTFIIB sequence at those four positions and the resulting constructs displayed reduced viability, temperature-sensitivity, and reduced activation at some promoters. Since cell viability was not abolished (as occurs upon substitution by wild type hTFIIB) other regions of TFIIB must contribute to inter-species functionality and transcription activation, a conclusion supported by the recovery of intragenic mutations outside helix B1 which suppress the point mutants described.

This chapter investigates the ability of AtTFIIB1 to interact with complexes derived from *Arabidopsis*, human and yeast TBP using EMSAs and fluorescence anisotropy. An estimate of the equilibrium binding constant for AtTFIIB1 interaction with the DNA-AtTBP complex is made, as well as measurements of AtTBP on- and off-rates with and without AtTFIIB1. Confirmation that AtTFIIB1 supports transcription is provided by *in vitro* reactions, and the HeLa origin of these reactions suggests compatibility with human transcription factors but not with the yeast system where plasmid shuffle tests produced non-viable cells.

Materials and Methods

Expression and Purification of Recombinant Proteins

A PCR amplification of *AtTFIIB1* was performed to attach a *Kpn*2I site before the first methionine and a *Sal*1 site in place of the stop codon using the primers 5AtIIB: 5' T-CATTTCCGGACATGTCGGATGCG 3' and 3AtIIBcore: 5' GAAACGGTCGACAGG-ACTTGACAGGT 3'. The PCR product was blunt-end ligated into the *Sma*I site of pUC19, and the resulting plasmid pAtTFIIB1 was digested with *Sst*I and *Sal*I. The resulting fragment was cloned into plasmid pET24b (Novagen) at those sites to produce a fusion protein carrying the T4 epitope at the N-terminus and a six-histidine tag at the C-terminus. Overnight cultures of *E. coli* BL21(DE3) were diluted 1:200 in LB medium with 0.01 mg/ml kanamycin and incubated at 37° C with shaking for 2.5 hr. After addition of 0.1 mM isopropyl β-D-thiogalactoside (IPTG), incubation continued with vigorous shaking at room temperature for 3-4 hr. Bacterial pellets were collected and sonicated in Ni binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 20 μg/ml pepstatin A, 20 μg/ml leupeptin, 1% aprotinin) and 0.1% NP-40. Aliquots (1.2 ml) of the cleared lysate were mixed with 100 μl of HisBind resin (50% slurry, Ni charged; Novagen) for 15-30 min at 4° C. Resin batches were pelleted by low-speed centrifugation and washed once with 1 ml Ni binding buffer and twice with Ni wash buffer (Ni binding buffer with 60 mM imidazole). Fifty to 100 μl of Ni elution buffer (0.3 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9) were added and the sample was rocked for 10-20 min at 4° C. Eluted proteins were stored at 4° C or flash frozen in liquid nitrogen after addition of

glycerol to 20% for storage at -70° C. pET vectors carrying histidine tagged cDNAs encoding AtTBP2, hTBP and yTBP (provided by Drs. Nam-Hai Chua and Robert Roeder, Rockefeller University) were used for TBP expression and purification as described above for AtTFIIB1. Total protein concentrations were determined with *dot*METRIC colorimetric strips (Geno Technology, Inc.) and compared to standard curves by spot densitometry using an IS1000 Digital Imaging System (Alpha Innotech Corp.). *E. coli* expressed proteins were electrophoresed on SDS-12% polyacrylamide gels along with Mid-Range Protein Markers (Promega) and visualized by Coomassie blue staining. The purity of recombinant proteins was determined by band peak integration with the IS1000 system. Western blot detection of TFIIB proteins is described in Chapter 4.

Electrophoretic Mobility Shift Assays

An oligonucleotide derived from the TATA region of the adenovirus major late promoter (AdMLP), 5' GGCTATAAAAGGGCTG 3', was ^{32}P end-labeled to 4.6 x 10^9 cpm/μg with T4 polynucleotide kinase and annealed with a 2-fold excess of bottom-strand oligomer. Probe (5 x 10^4 cpm) was added to EMSA reactions containing BS buffer (10 mM HEPES pH 7.9, 4 mM $MgCl_2$, 5 mM NH_3SO_4, 50 mM KCl, 0.2 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol) and incubated with TBP or TBP+TFIIB at 37° C for 45 min. Reactions were electrophoresed (125 V at room temperature) on 4% polyacrylamide (30:1) gels containing 10% glycerol in either TGM buffer (25 mM Tris, 100 mM glycine, 4 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT) or 0.5X TBE buffer supplemented with the same concentrations of Mg, glycerol and DTT.

Fluorescence Polarimetry

A second AdMLP TATA probe was synthesized (CyberSyn) carrying sequences through the transcription start site and modified with a 3' fluorescein tag on the bottom strand: 5' GGGGGGCTATAAAAGGGGGTGGGGGCGCGTTCGTCCTCACT 3'. A competitor DNA was also created by annealing oligonucleotides with the same sequences but lacking the fluorescein tag. Binding reactions contained BS buffer with no glycerol or β-mercaptoethanol, 0.5 nM DNA probe, and TBP or TBP+TFIIB in a volume of 0.35 ml at ambient temperature in the sample chamber (28° C after warm-up). Data were collected from an SLM/Aminco polarizing luminescence spectrometer (Series 2, Aminco Bowman) set for excitation at 494 nm, bandpass 4 and detection of emission at 520 nm, bandpass 16. Data collected under equilibrium conditions are the average of 10 one-second measurements, and kinetic experiments were measured at five-second intervals for 500-1000 sec. Anisotropy values were calculated after subtraction of background fluorescence, which was measured in parallel reactions that received identical experimental manipulation but contained no fluorescein-labeled DNA. The data in Fig. 3-3 are raw signal traces from the spectrometer without background correction and were collected as polarization units (P) to emphasize this difference. Polarization is calculated as

$$P = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}}$$

and the mathematical maximum for P is 0.5. Binding reactions were analyzed by non-linear regression with curve fitting (SigmaPlot, Jandel Scientific).

In vitro Transcription

HeLa crude nuclear extracts prepared in the laboratory of Dr. J. B. Flanegan (University of Florida) (Dignam *et al.*, 1983) were incubated on ice for 30 min with IgG monoclonal antibodies to hTFIIB (Promega) at 400 μg per ml extract. Sepharose linked to anti-IgG antibody (Sigma) was then added, incubated and centrifuged for 2 min at 500 x g to deplete hTFIIB from the extract. Reactions were assembled containing transcription buffer (10 mM HEPES pH 7.9, 50 mM KCl, 0.1 mM EDTA, 0.25 mM DTT, 10% glycerol), 3 mM $MgCl_2$, 0.4 mM each of ATP, CTP and GTP, 100 ng template DNA, and 1 μl of α-^{32}P UTP (high specific activity, 10 mCi/ml, Amersham) in a final volume of 30 μl. The supercoiled plasmid used as a transcription template carries the cytomegalovirus (CMV) immediate early promoter fused to a hammerhead ribozyme sequence (Batt, 1996) so that template derived RNA will be consistently self-cleaved at the same 3' terminus. Reactions also contained 5 μl of HeLa extract (6.3 mg/ml total protein) or an equivalent amount (by protein mass) of depleted extract plus recombinant TFIIB, and were incubated at 30° C for 60 min before adding 175 μl of stop mix (0.3 M Tris-HCl pH 7.4, 0.3 M sodium acetate, 0.5% SDS, 2 mM EDTA, 3 μg/ml tRNA). Extraction with phenol:chloroform:isoamyl alcohol was followed by ethanol precipitation and resuspension of the RNA pellet in nuclease-free water. Samples were electrophoresed on a 6% denaturing polyacrylamide (40:1) gel. Autoradiographs were quantified by densitometry on the IS1000 system.

Yeast Plasmid Shuffle for TFIIB

AtTFIIB1 cDNA was subcloned as an *Nde*I-*Not*I fragment from pET24b into two yeast expression vectors. p1210A is a high copy number plasmid driving expression from the strong yeast Adh1 promoter, and pLC1210 is a low copy number version expressing AtTFIIB1 from the wild type yeast TFIIB promoter. Both plasmids were provided by Dr. Jun Ma (University of Cincinnati), and in his laboratory Dr. Shruti Shaw performed the yeast transformations to replace yTFIIB with AtTFIIB1 as described (Shaw *et al.*, 1996).

Results

Proteins produced by *E. coli* expression and elution from HisBind resin ranged from 50-80% purity and from 0.2-0.5 μg/μl in concentration (Fig. 3-1A). EMSA gels for AtTBP and hTBP interaction with the TATA site were best resolved in supplemented TGM buffer. AtTBP binding was specific for the TATA oligomer probe and stable in the presence of non-specific competitors (Fig. 3-1B). Addition of AtTFIIB1 to the AtTBP reaction produced a second band with slower mobility (Fig. 3-1B lane 5) that was not present after addition of HisBind purified *E. coli* lysate from a culture not expressing AtTFIIB1 (data not shown). A similar reaction combining hTBP and AtTFIIB1 did not show a discrete second band or any other obvious changes in hTBP binding (Fig. 3-1C).

Yeast TBP showed poor EMSA resolution in the TGM buffer system, so gels were run in supplemented 0.5X TBE. Increasing concentrations of yTBP shifted a greater proportion of the probe (Fig. 3-2A), and three of these concentrations were tested for

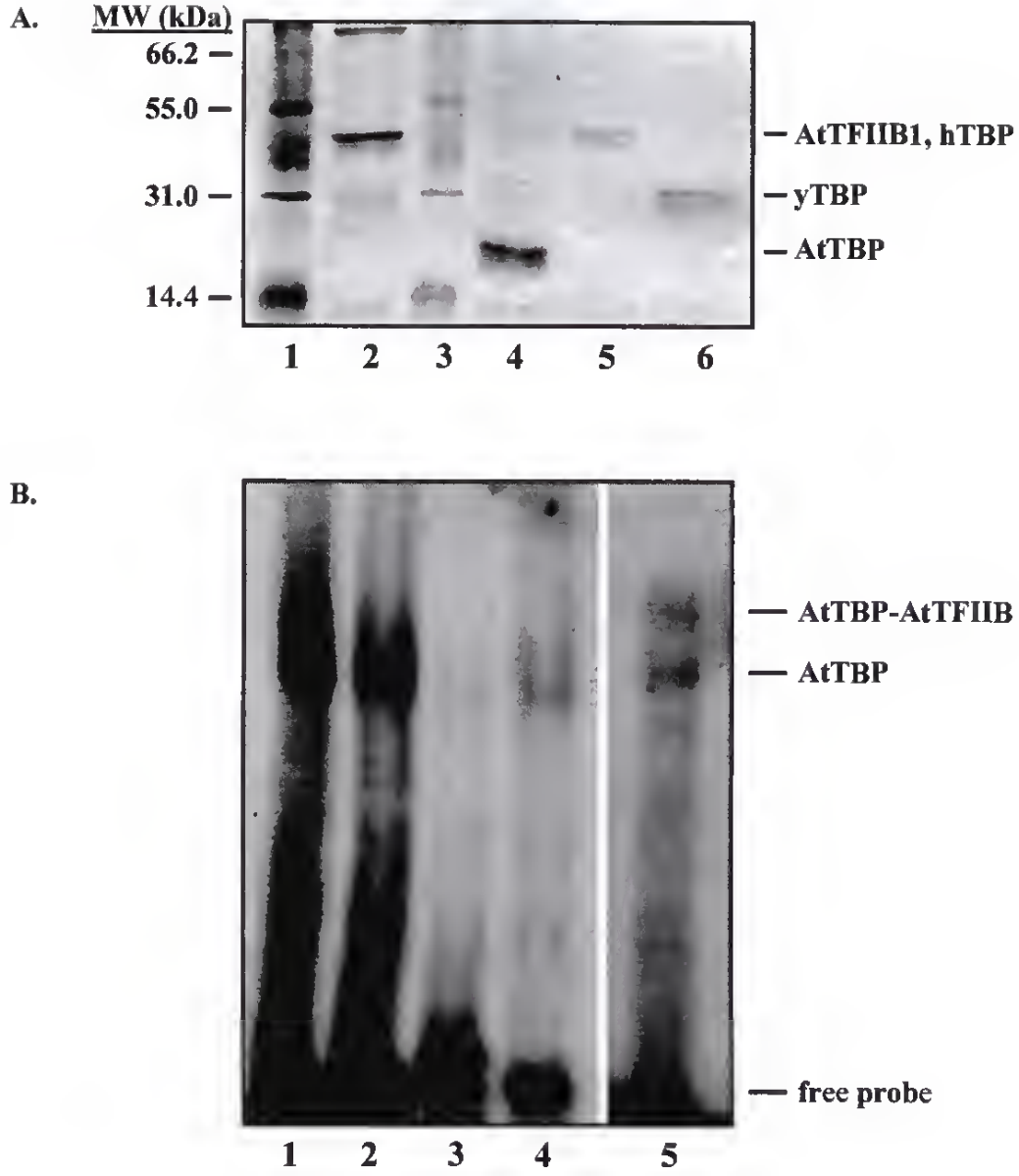


Figure 3-1. Purification and interaction of TBP and AtTFIIB1.
A. Coomassie stained recombinant proteins (10 µl) were electrophoresed on a 12% SDS-polyacrylamide gel. Lanes: 1) 1.5 µg of marker, 2) AtTFIIB1, 3) 0.5 µg of marker, 4) AtTBP, 5) hTBP, 6) yTBP. **B.** EMSAs detect TATA binding by 1 µg of AtTBP (lane 1) which is stable in 1 µg of poly d(I)d(C) + 0.5 µg of tRNA (lane 2), but specifically competed by 100-fold excess of unlabeled TATA oligomer (lane 3). AtTBP (0.1 µg) also binds (lane 4) and shows enhanced interaction and a larger complex with addition of 0.2 µg of AtTFIIB1 (lane 5).

C.

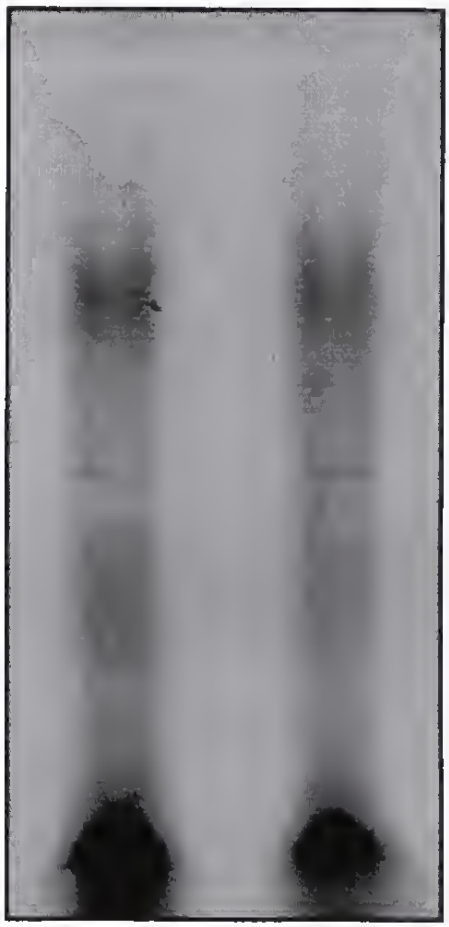


Figure 3-1 -- continued
C. EMSAs detect similar TATA binding by 0.2 μg of hTBP without (lane 1) or with (lane 2) 0.4 μg of AtTFIIB1.

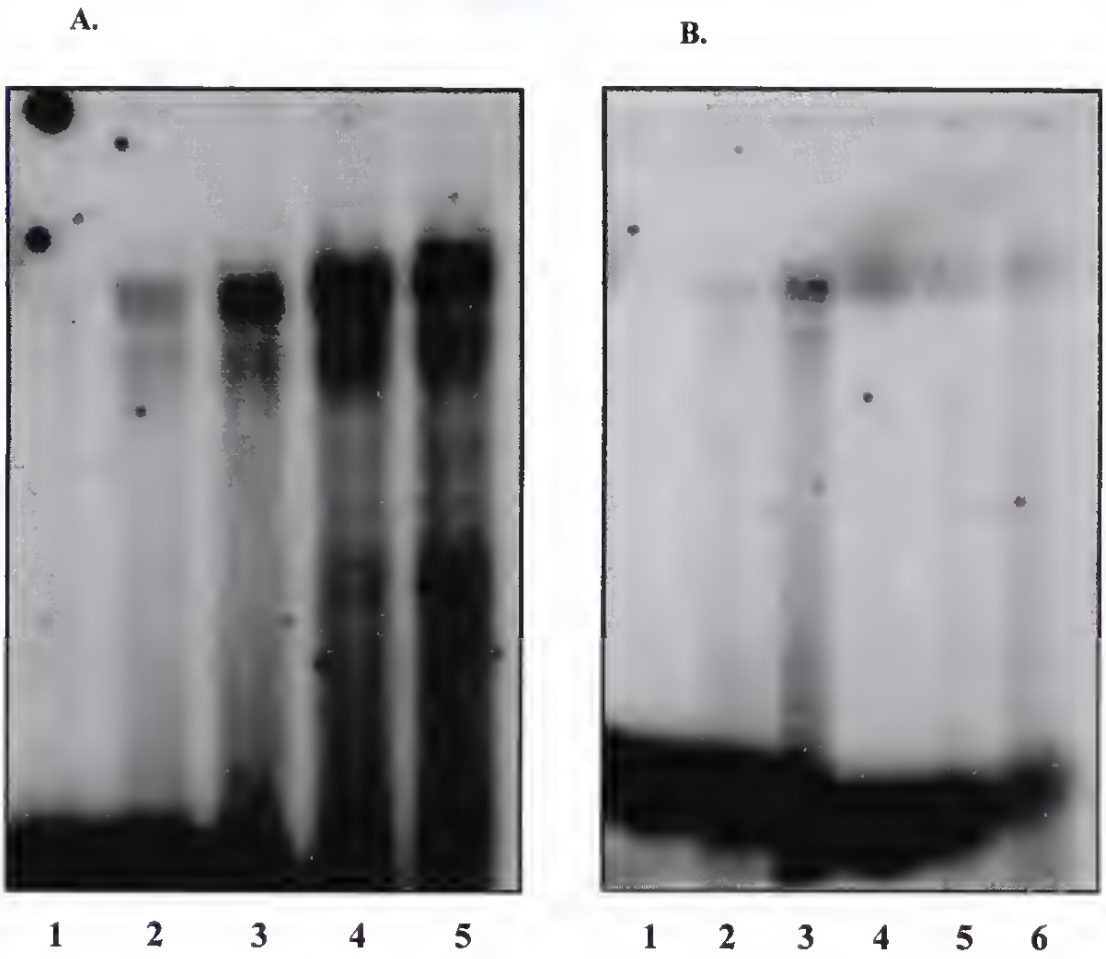


Figure 3-2. Interaction of yTBP with the TATA box and AtTFIIB1.
A. Gels in 0.5X TBE resolve the products of EMSA reactions with the TATA probe and varying amounts of yTBP. Lanes: 1) 30 ng, 2) 60 ng, 3) 0.12 μg, 4) 0.24 μg, 5) 0.48 μg.
B. Lanes 1-3 are replicates of those in (A.), lanes 4-6 are replicates with 60 ng (lane 4), 0.12 μg (lane 5), and 0.24 μg (lane 6) of AtTFIIB1 added.

AtTFIIB1 binding. With a limiting quantity of yTBP insufficient to shift a detectable amount of probe, addition of AtTFIIB1 enhanced yTBP binding significantly (Fig. 3-2B, lanes 1 and 4) and with a wider range of mobility. At higher yTBP concentrations binding appeared to be reduced rather than enhanced, but with a consistent reduction in the amount of free probe detected.

The EMSA analysis suggested AtTFIIB1 does have affinity for AtTBP and yTBP proteins, but variable conditions were required and produced results which were difficult to quantify. Fluorescence polarimetry was therefore tested to determine whether changes in anisotropy could be detected for TBP binding to the TATA box and for subsequent binding of AtTFIIB1. Fig. 3-3 is a trace of typical polarization data obtained in an experiment monitoring AtTBP binding to fluorescein-labeled TATA box DNA. Free probe rotates quickly and showed little polarization upon addition to the cuvette at 65 sec, but introducing AtTBP at 100 sec immediately formed a larger volume complex that slowed rotation and increased polarization to more than 0.3 P (Fig. 3-3A). A 10-fold excess of unlabeled competitor TATA reduced the binding signal (Fig. 3-3B, 500 sec), and 100-fold excess of competitor DNA further reduced binding to a level near that of free probe (700 sec). A parallel reaction to test for non-specific binding produced a maximum signal around 0.25 P and was not affected by 100-fold excess poly d(I)d(C) (Fig. 3-3C).

TBP binding reactions were measured at equilibrium, corrected for background fluorescence and plotted in anisotropy units for comparisons of AtTFIIB1 interaction. Where indicated, total F is the total fluorescence emitted and should remain at a steady level for different treatments within an experiment. Variation of this control indicates an

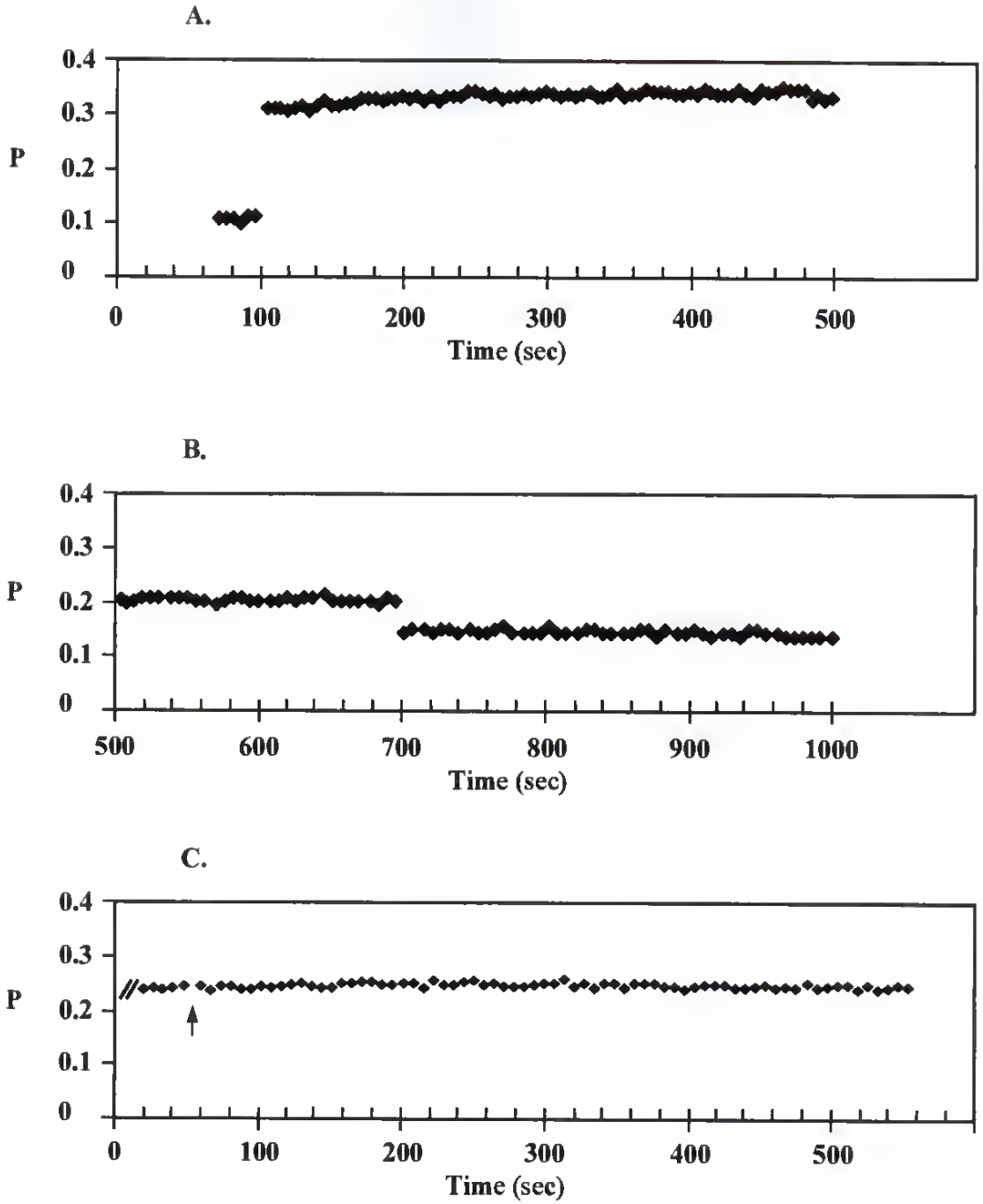


Figure 3-3. Fluorescence polarization vs. time for AtTBP interaction with the TATA box. **A.** Fluorescein-tagged TATA probe (1 nM) exhibited 0.1 polarization units (P) when added to the binding reaction at 65 sec, and addition of 0.5 μg of AtTBP at 100 sec created a complex with over 0.3 P. **B.** Addition of 10-fold excess unlabeled TATA competitor to the reaction in (A.) at 500 sec reduced the binding signal to 0.2 P, and a further reduction occurred when the competitor was increased to 100-fold excess over probe at 700 sec. **C.** A reaction similar to (A.) reached equilibrium at 0.25 P before addition of poly d(I)-d(C) to 100 nM (arrow).

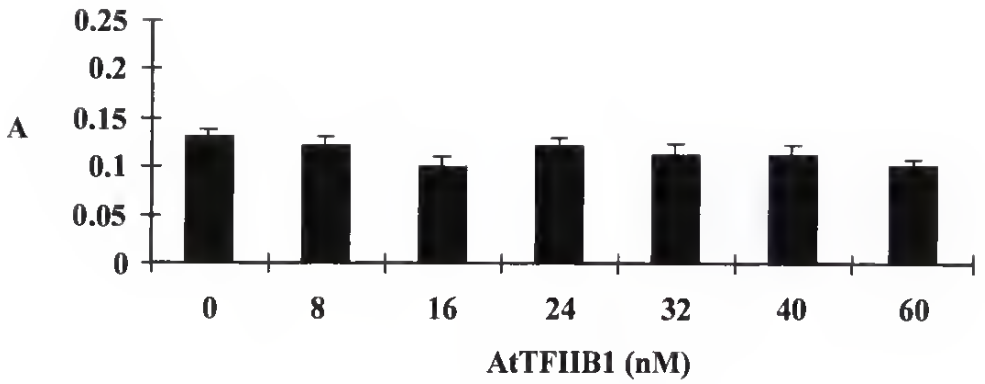


Figure 3-4. Fluorescence anisotropy of the TATA box with AtTFIIB1.
TATA probe (0.5 nM) was combined with the indicated concentrations of AtTFIIB1 and incubated 15 min. Average anisotropy with standard deviation of ten measurements is shown.

alteration of fluorescence intensity from the fluorescein tag that may indicate large scale conformation changes or interference from excess protein levels. The results of a second experimental control are shown in Fig. 3-4 in which AtTFIIB1 alone showed no binding affinity for the TATA probe. Figs. 3-5 and 3-6 show data for three TBP homologs obtained from progressive assembly of two parallel binding reactions, measured after 10 min incubations of each assembly step to ensure the components are at equilibrium. Assembly steps 1-3 were in a double-volume reaction and represent free probe (1), probe-TBP (2), and probe-TBP plus 100-fold excess competitor DNA (3). The addition of competitor oligomer incorporated most free TBP into TATA complexes so that any further change in anisotropy was due to TFIIB binding and not simply an equilibrium shift toward more TBP binding DNA. The reactions were split following step 3 to test AtTFIIB1 and control treatments. Fig. 3-5 shows that addition of equal or 2-fold amounts of AtTFIIB1 relative to AtTBP slightly increased the anisotropy and a 3-fold excess of AtTFIIB1 showed a significant anisotropy increase, suggesting that TFIIB was binding to the TATA-TBP complex. The parallel reaction received equal or excess amounts of BSA rather than AtTFIIB1. The results (Fig. 3-5 open bars) indicated that the increased anisotropy observed with AtTFIIB1 was specific for ternary complexes and was not due to general TBP stabilization by higher protein concentrations. In fact, extra non-specific protein appears to de-stabilize TBP binding, an effect previously reported for much higher BSA concentrations in yTBP footprint experiments (Imbalzano *et al.*, 1994). It is unclear why BSA induced less anisotropy than observed for free probe in this experiment. In Fig. 3-6, AtTFIIB1 slightly increased anisotropy with hTBP but not to the degree seen

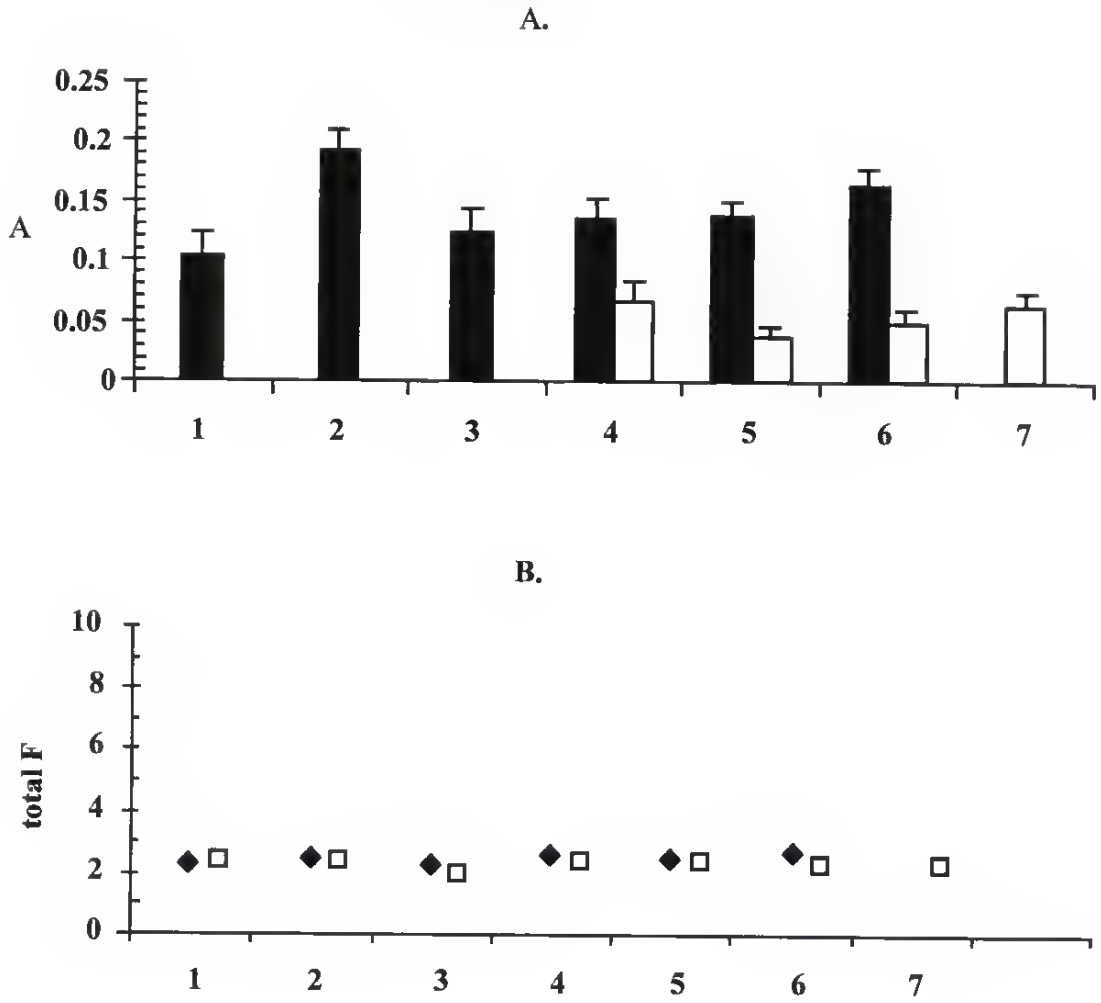


Figure 3-5. Equilibrium anisotropy of TATA-AtTBP-AtTFIIB interaction.
A. A double-volume reaction containing 0.5 nM TATA probe was incubated with 15 nM AtTBP, followed by 50 nM competitor oligomer. Anisotropy (A) was measured for free probe (1), probe+AtTBP (2), and probe+AtTBP+competitor (3). The reaction was then equally divided into two cuvettes. Sequential aliquots of AtTFIIB1 were added to one treatment (filled bars) to final concentrations of 20 nM (4), 40 nM (5), and 60 nM (6). The second reaction (open bars) received no AtTFIIB1, and BSA was added to final concentrations of 20 nM (4), 60 nM (5), 120 nM (6), and 240 nM (7). Each assembly step was incubated for 10 min before collecting 10 measurements of A. Average A with standard deviations are shown. **B.** Total fluorescence intensity for the AtTFIIB1 reaction (filled diamonds) and BSA reaction (open squares) remained constant. All protein concentrations reported for AtTBP and AtTFIIB in figures for this chapter are adjusted for binding activity, as assayed in Fig. 3-10.

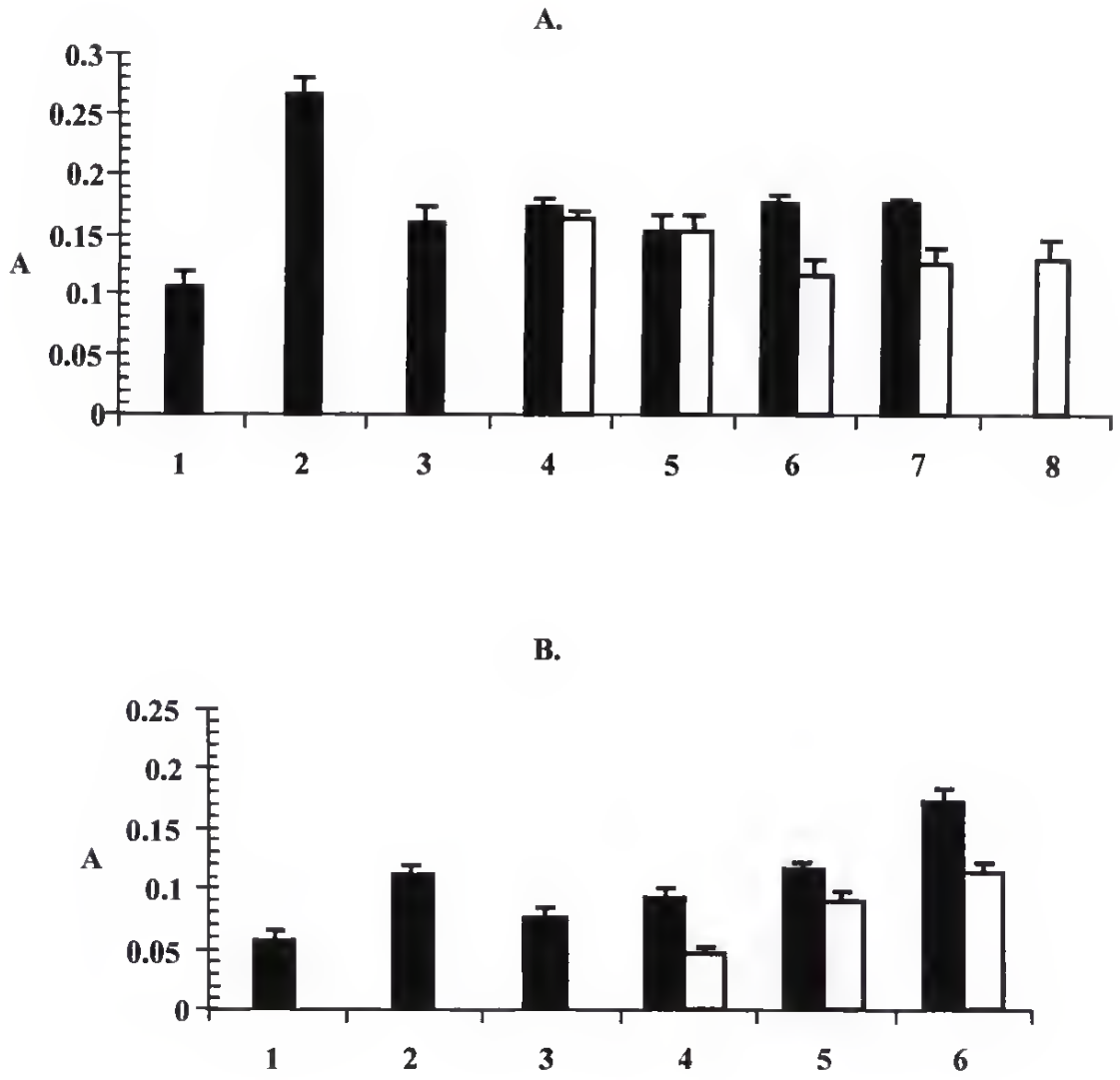


Figure 3-6. Equilibrium anisotropy of AtTFIIB1 interactions with hTBP and yTBP. **A.** Reactions were assembled as in Fig. 3-5 substituting hTBP for AtTBP. AtTFIIB1 was added (filled bars) to final concentrations of 20 nM (4), 40 nM (5), 60 nM (6), and 80 nM (7). The BSA series (open bars) received 60 nM (4), 120 nM (5), 180 nM (6), 240 nM (7), and 300 nM (8) non-specific protein. **B.** yTBP was combined with AtTFIIB1 (filled bars) at concentrations listed in Fig. 3-5, or with BSA (open bars) at 60 nM (4), 120 nM (5), and 180 nM (6).

with AtTBP. yTBP appears to have high affinity for AtTFIIB1, but BSA also significantly increased anisotropy with increasing concentration. No change in total fluorescence was observed in these experiments (Fig. 3-4 and data not shown).

To determine whether AtTFIIB1 affects the K_d for TBP-DNA binding in these reactions, binding curves were measured and are plotted in Figs. 3-7 to 3-9. Increasing concentrations of TBP with and without a 3-fold excess of AtTFIIB1 were added to 0.5 nM TATA probe and allowed to reach equilibrium. The inflection points of the resulting curves are the apparent K_d values. The presence of AtTFIIB1 raised anisotropy values throughout the curve and increased AtTBP affinity for probe DNA by 1.9-fold (Fig. 3-7). AtTFIIB1 also increased anisotropy with hTBP (Fig. 3-8), but the K_d appears unchanged. The fitted curves for yTBP binding were linear, rather than sigmoidal, which is usually interpreted as a non-specific interaction (Fig. 3-9). Attempts to detect non-specific DNA binding by poly d(I)d(C) competition showed no effect on yTBP or hTBP (data not shown).

A more detailed investigation of AtTFIIB1 influences on AtTBP was conducted by measuring the on- and off-rates for DNA binding. A plot of binding vs. time indicated that AtTBP quickly associated with the TATA box with the probe half occupied in less than five seconds (Fig. 3-10A). Repeating the assay with AtTFIIB1 present had little effect, and BSA reduced overall binding but not the rate of association. Dissociation, however, was altered by AtTFIIB1 (Fig. 3-10B and C) which obviously decreased the AtTBP off-rate after addition of 200-fold excess competitor DNA. The k_d for each fitted curve was calculated by plotting the natural log of $(A_{obs} - A_{min})/(A_{max} - A_{min})$ vs. time,

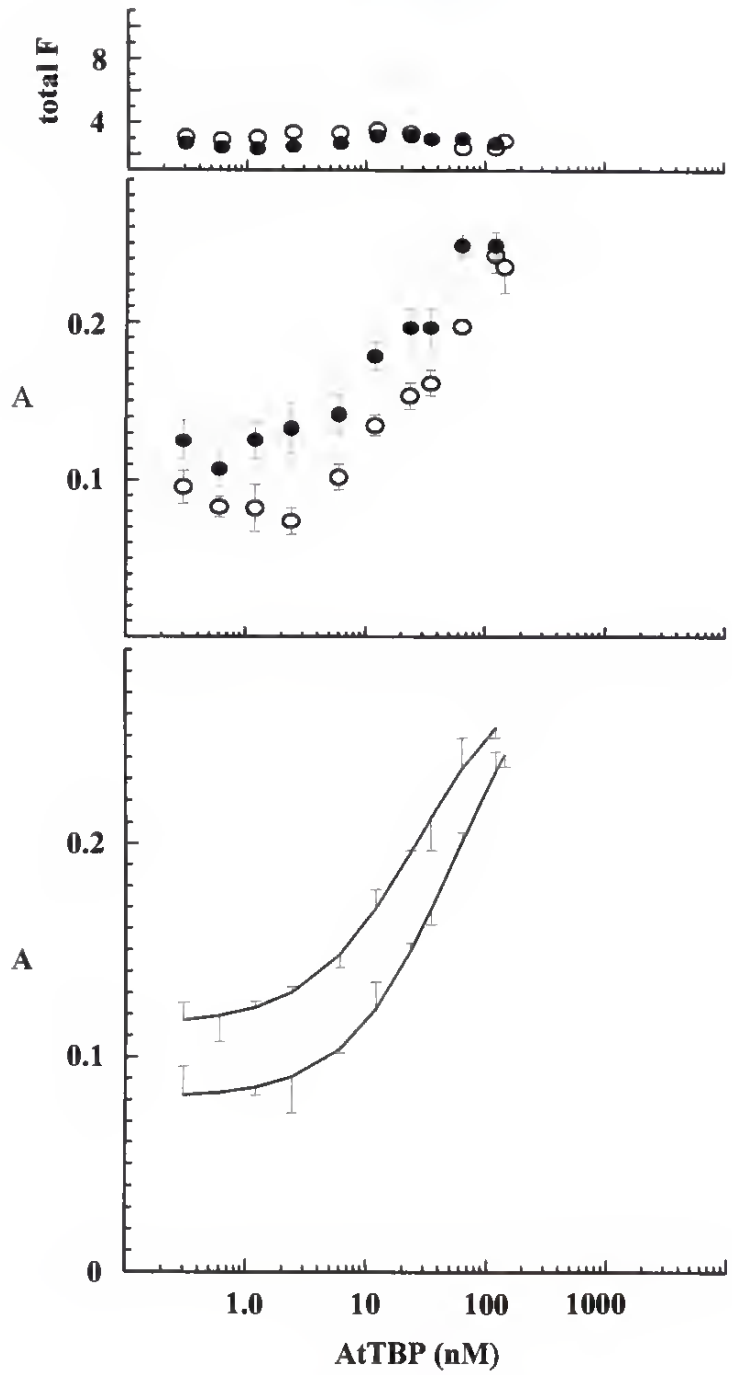


Figure 3-7. TATA binding titration of AtTBP with and without AtTFIIB1. Increasing concentrations of AtTBP were bound to 0.5 nM TATA probe and allowed to reach equilibrium in the absence (open circles) or presence (filled circles) of AtTFIIB1 at three-fold higher concentrations. The average and standard deviation of ten anisotropy measurements per reaction are indicated (middle panel), as well as total fluorescence (top). Fitted curves were generated by non-linear regression (bottom), and residuals from the equation for each curve are shown as vertical error bars.

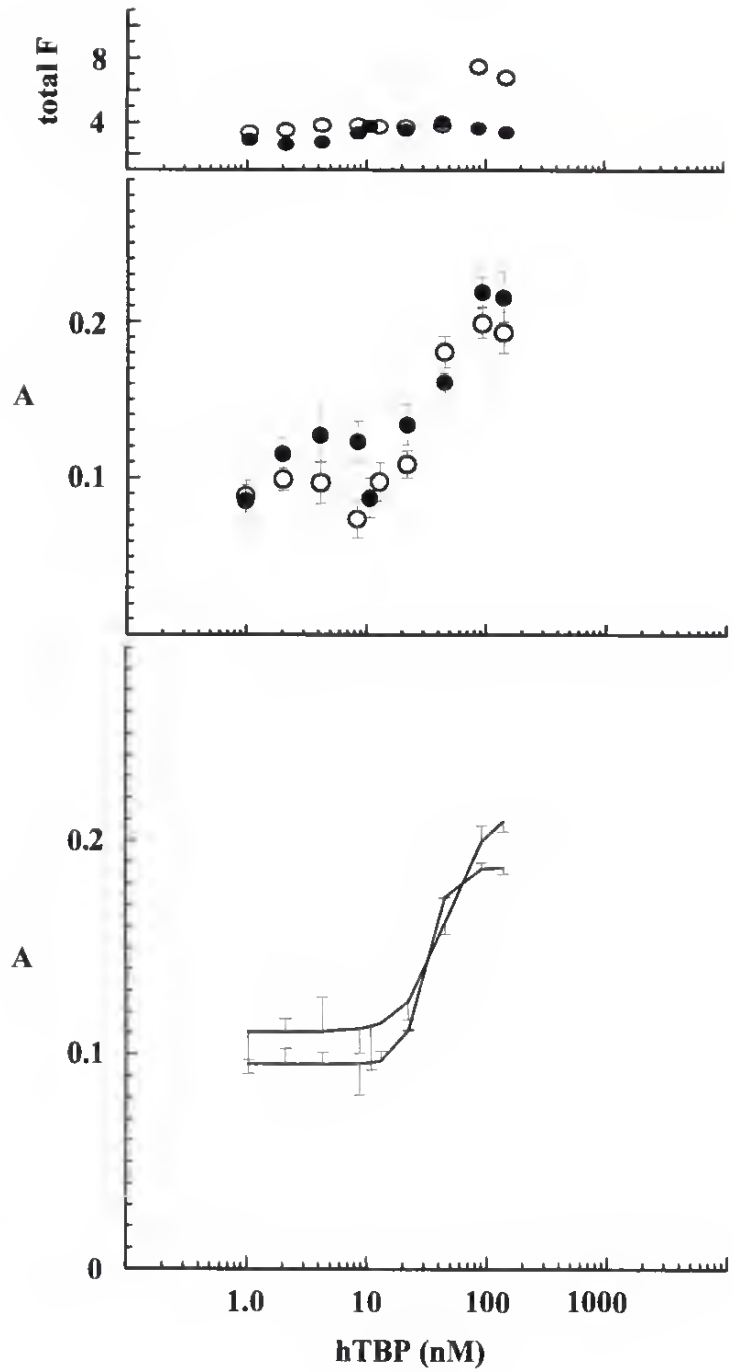


Figure 3-8. TATA binding titration of hTBP with and without AtTFIIB1. Increasing concentrations of hTBP were bound to 0.5 nM TATA probe and allowed to reach equilibrium in the absence (open circles) or presence (filled circles) of AtTFIIB1 at three-fold higher concentrations. Data are plotted as described in Fig. 3-7.

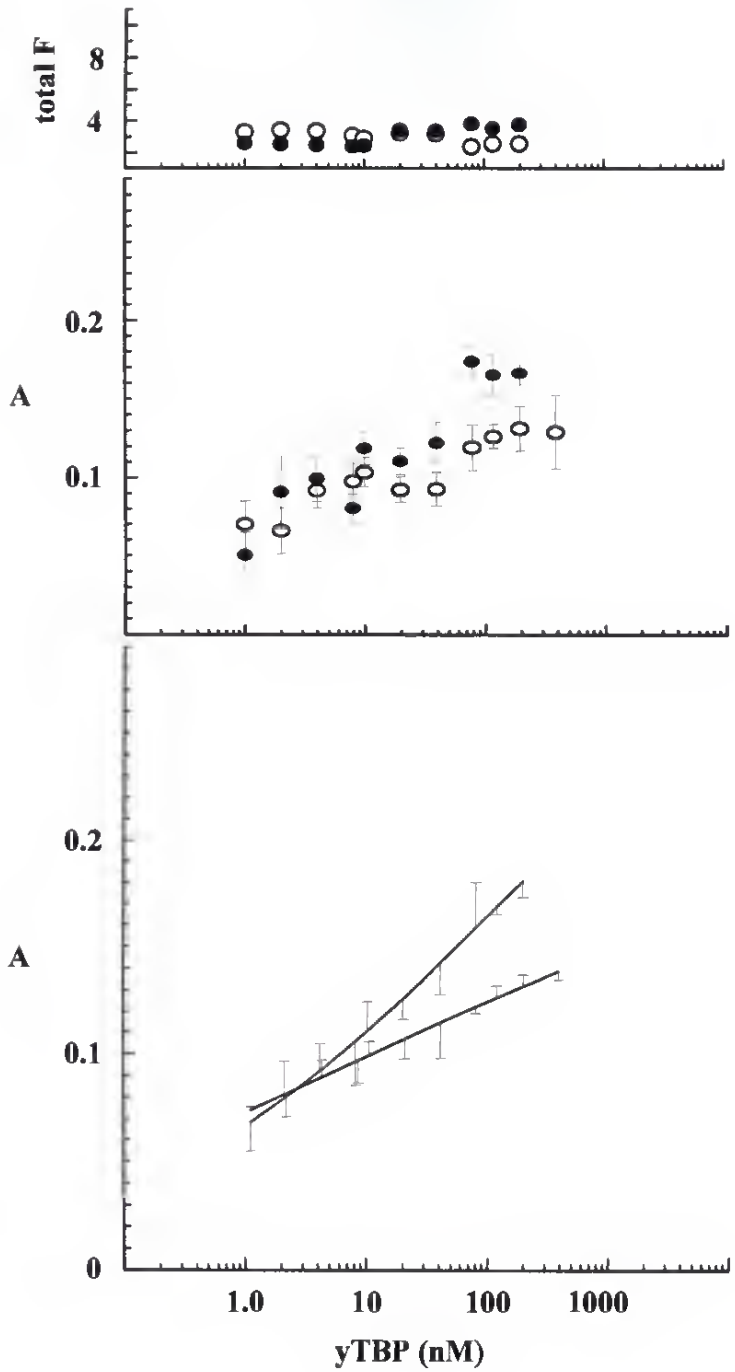


Figure 3-9. TATA binding titration of yTBP with and without AtTFIIB1. Increasing concentrations of yTBP were bound to 0.5 nM TATA probe and allowed to reach equilibrium in the absence (open circles) or presence (filled circles) of AtTFIIB1 at three-fold higher concentrations. Data are plotted as described in Fig. 3-7.

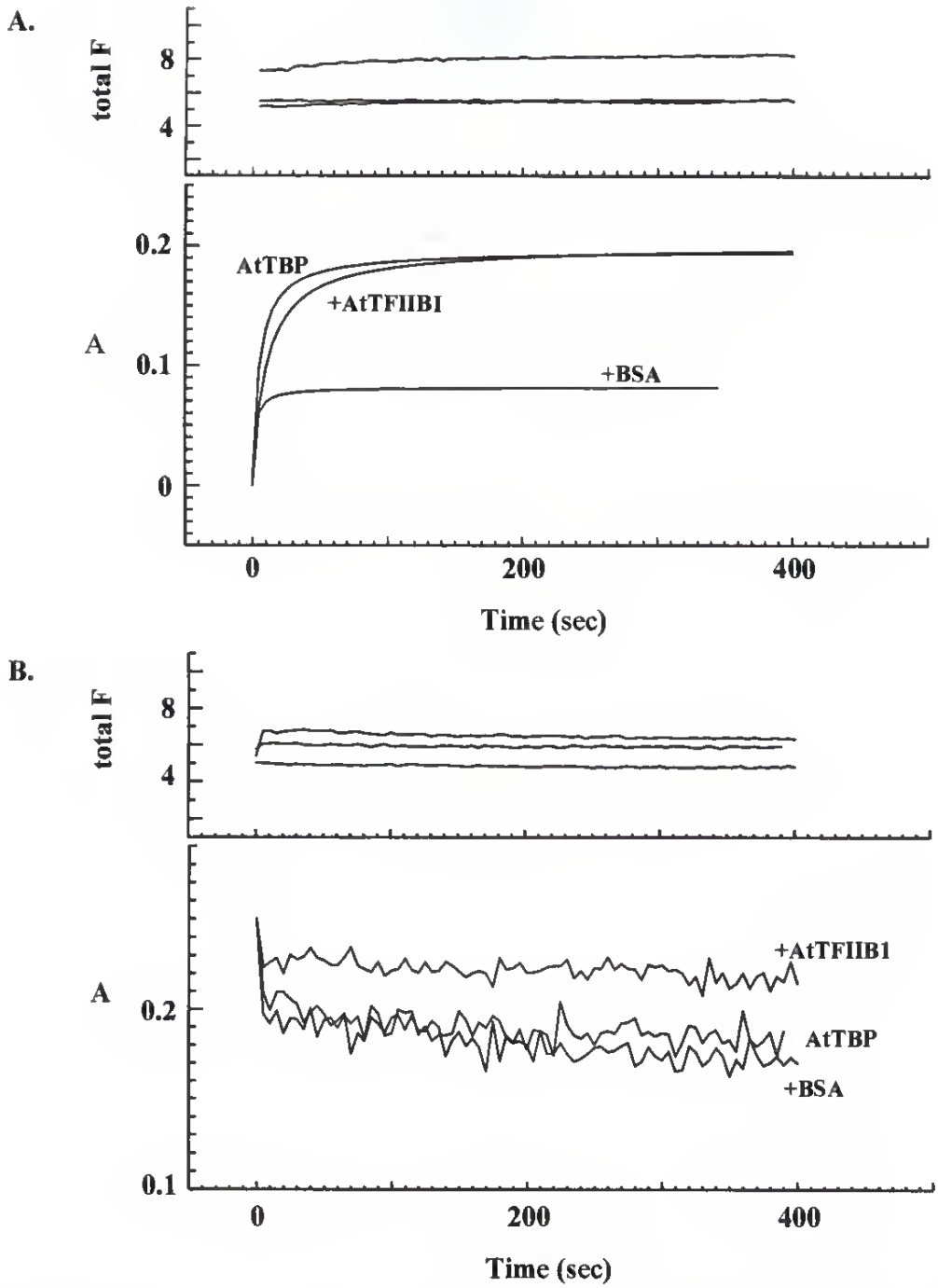


Figure 3-10. Rates of AtTBP association and dissociation from the TATA box with and without AtTFIIB1.

A. A reaction containing 0.5 nM TATA probe was adjusted to 47 nM AtTBP and anisotropy was measured at five second intervals. The assay was then repeated in the presence of 84 nM AtTFIIB1 or 84 nM BSA. Curve-fitting equations for AtTBP and AtTBP+AtTFIIB1 were normalized to the same plateau to compare curve shapes.

B. AtTBP (35 nM) was pre-equilibrated with 0.5 nM TATA probe, or probe and 75 nM AtTFIIB1 or BSA, before addition of competitor oligomer to 100 nM at time 0.

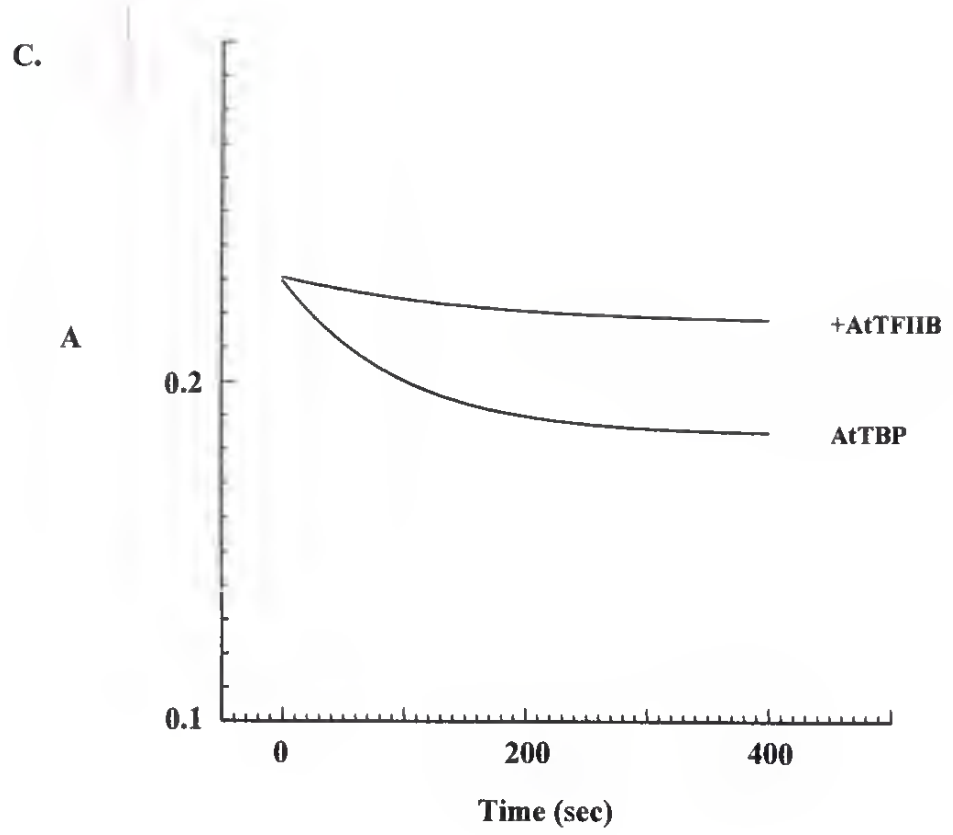


Figure 3-10 -- continued
C. Fitted curves for the data collected in (B.) were calculated after normalization to equalize the binding signals at 0 sec.

where A_{obs} is the anisotropy at each time point and A_{max} and A_{min} are the maximum and minimum values observed during the assay (Beacon Applications Guide, 1995). The absolute value of the slope of the resulting line is the k_d which for AtTBP alone was 1.01 x 10^{-2} sec^{-1} with a half-time ($t_{1/2}$) of 69 sec. Addition of AtTFIIB1 reduced the k_d to 6.9 x 10^{-3} sec^{-1} ($t_{1/2}$ = 100 sec), a 1.5-fold decrease in the off-rate.

In order to estimate the apparent K_d for AtTFIIB1 binding to the DNA-AtTBP complex, the binding activity of the *E. coli* expressed proteins was first determined. Probe (0.5 nM) was mixed with 50 nM TATA box competitor and AtTBP was added until the anisotropy signal plateaued, indicating saturation of all TATA binding sites (Fig. 3-11A). The half-maximal binding point on this curve indicates that 42.7 pmol (955 ng) of AtTBP was required to occupy 8.8 pmol (222 ng) of DNA oligomer, or 20.6% specific binding activity for AtTBP assuming binding occurs as a monomer. This is similar to the activity measured for recombinant yTBP (Hoopes *et al.*, 1992). A second reaction was performed with the same DNA concentration, 11 pmol of AtTBP (246 ng), and increasing amounts of AtTFIIB1 (Fig. 3-11B). Since there should have been enough excess DNA present to occupy most of the active AtTBP, the inflection point of this curve indicates 21.3 pmol (728 ng) of AtTFIIB1 was required to bind 5.5 pmol of DNA-AtTBP, or a binding activity of 25.7% for AtTFIIB1. This calculation again assumes monomeric binding and also no significant affinity of AtTFIIB1 for the inactive, non-DNA associated AtTBP which would reduce the activity observed. There are no reported measurements of TFIIB affinity for TBP in the absence of TATA DNA using recombinant proteins, but the two factors do not co-purify from nuclear extracts (Buratowski *et al.*, 1989; Maldonado *et al.*, 1990). A final titration was conducted with 0.5 nM labeled probe, 8 nM active AtTBP,

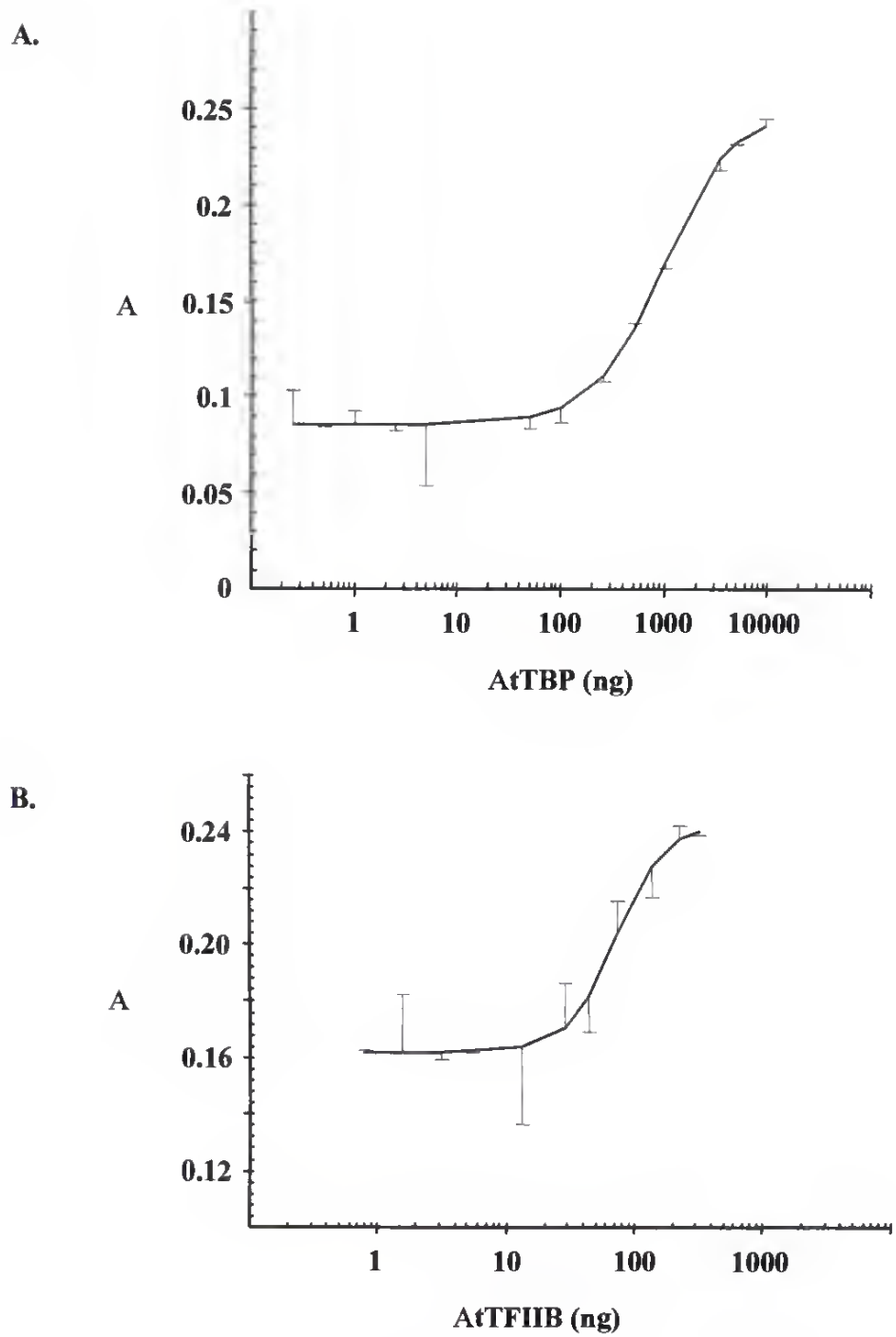


Figure 3-11. Titration of binding activity for recombinant AtTBP and AtTFIIB. **A.** Unlabeled TATA oligomer (50 nM) was mixed with 0.5 nM TATA probe. Average anisotropy was measured after 10 min incubations with sequentially added aliquots of AtTBP. Data are shown after curve fitting and residuals are indicated. **B.** A reaction containing 50 nM unlabeled TATA oligomer, 0.5 nM TATA probe, and 31 nM AtTBP (246 ng) was pre-equilibrated before addition of AtTFIIB1 and analysis as in (A.).

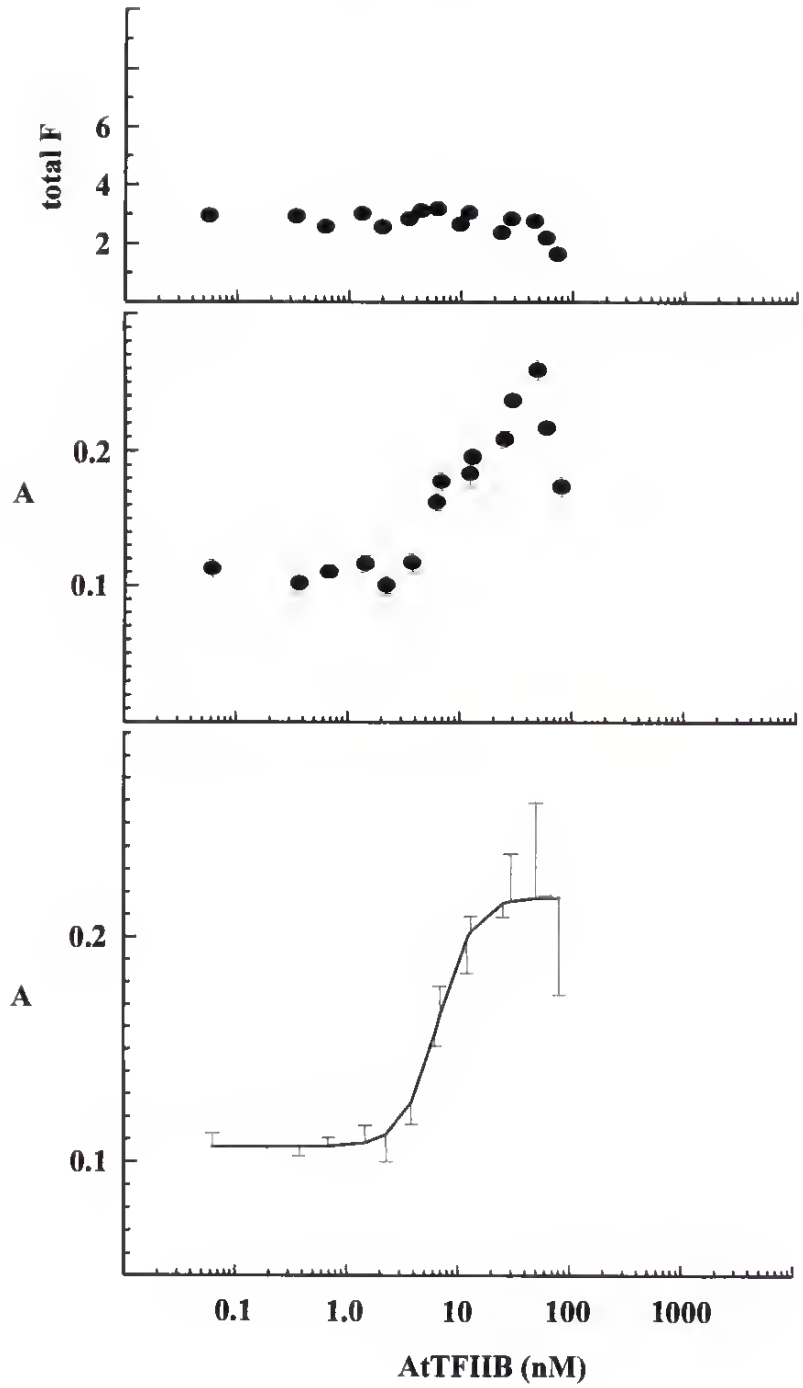


Figure 3-12. Determination of equilibrium binding constant for AtTFIIB1 interaction with DNA-AtTBP.
TATA probe (0.5 nM) and 8 nM AtTBP were at equilibrium before addition of AtTFIIB1 aliquots for additional 10 min incubations. Data were analyzed as in Fig. 3-7.

and a range of concentrations of AtTFIIB1 to generate the binding curve depicted in Fig. 3-12. Under these conditions probe is limiting for complex formation and the inflection point occurs at the apparent K_d of 7.1 nM for AtTFIIB1 interaction with DNA-AtTBP.

The ability of AtTFIIB1 to interact with hTBP and support *in vitro* transcription was tested using HeLa nuclear extracts. Transcripts from the CMV promoter-ribozyme reporter were resolved on a 6% sequencing gel as seven bands one nucleotide apart when untreated extract was used (Fig. 3-13A, lane 1), and depletion of the extract with anti-hTFIIB antibodies reduced transcription in lane 2 to 35% relative to the untreated reaction. The background bacterial proteins present after purification of *E. coli* lysates did not influence the observed transcription (lane 3). Addition of increasing amounts of recombinant hTFIIB or AtTFIIB1 to the depleted extract partially restored activity by increasing accumulation of three transcripts with adjacent start sites. A site-directed mutagenesis reaction was used to create a single amino acid substitution in AtTFIIB1 at residue 43 (glutamic acid changed to arginine) in the conserved region. The analogous mutation in yTFIIB altered the start site at several promoters *in vivo* (Pinto *et al.*, 1994), but had no effect on AtTFIIB1 in this assay. The values shown for percent relative transcription for each treatment in Fig. 3-13A were derived from six replicate reactions that contained 400 ng of the various TFIIB proteins tested. The seemingly impaired ability of hTFIIB to restore transcription may have been due to carryover of the antibody used for immuno-depletion, which bound recombinant hTFIIB but not AtTFIIB1 (Fig. 3-13B). The anti-IgG resin used to deplete the extract was assumed to be present in sufficient concentration to prevent such carryover; it is also possible that *E. coli* expressed hTFIIB is less active than AtTFIIB1.

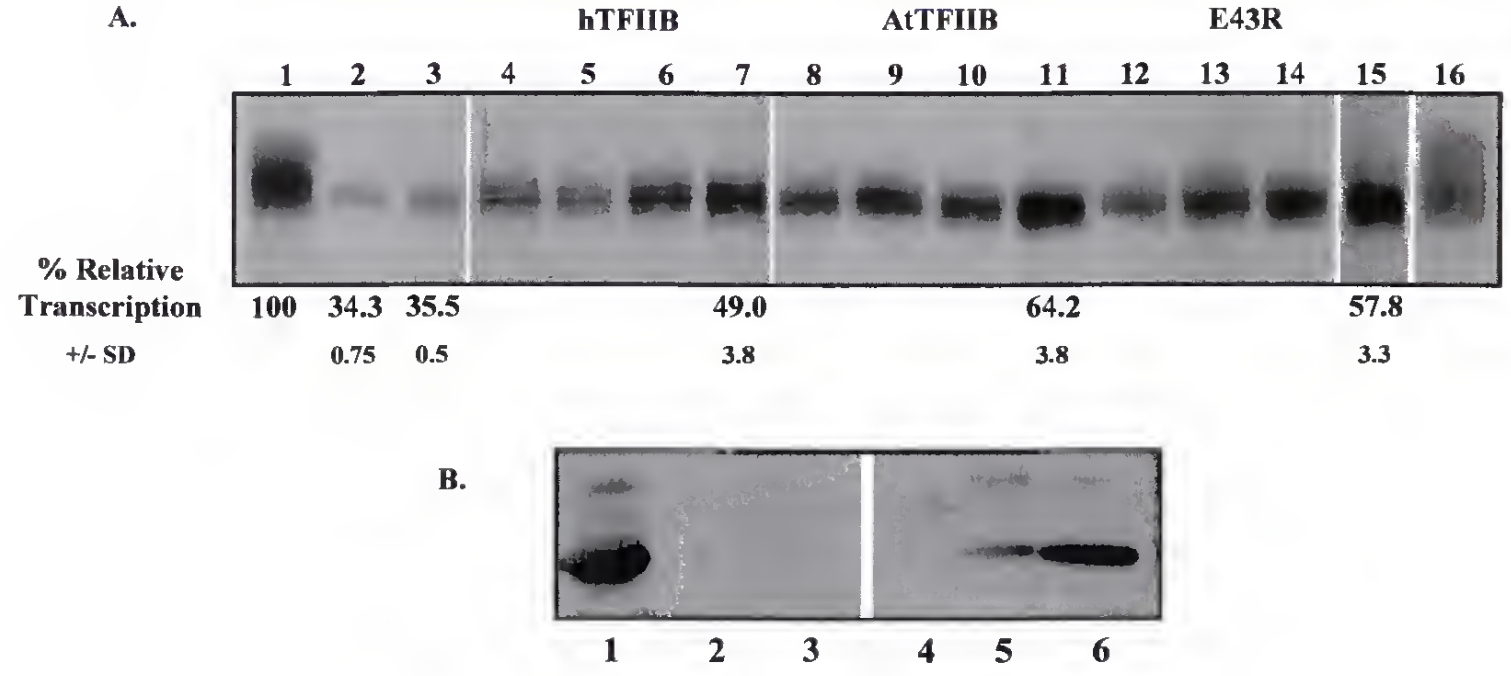


Figure 3-13. AtTFIIB1 supports *in vitro* transcription using HeLa nuclear extracts.
A. *In vitro* transcription from a CMV promoter produces ribozyme RNA transcripts centered around 209 nucleotides in length. Five µl of HeLa nuclear extract (lanes I and 16) or an aliquot of depleted extract containing an equivalent amount of total protein was used for each reaction. Extract depleted of hTFIIB (lane 2) was supplemented with control *E. coli* lysate (lane 3) or 50, 100, 200, and 400 ng purified hTFIIB (lanes 4-7). The same amounts of purified AtTFIIB1 were also tested (lanes 8-11) as well as the AtTFIIB1 mutant E43R (lanes 12-15). Six replicates of the reactions containing 400 ng recombinant protein were performed (not shown), and the levels of transcription relative to untreated extract are indicated as average percentages with standard deviations. **B.** In lanes 1-3, the monoclonal anti-hTFIIB antibody used for immuno-depletion detects recombinant hTFIIB (lane 1) but not AtTFIIB1 (lane 2) or its mutant E43R (lane 3). Lanes 4-6 are a replicate Western blot but probed with antibody against the T7 epitope which is fused to AtTFIIB1 and its mutant E43R in the pET24b expression vector (see also Fig. 4-6A).

A plasmid substitution experiment was conducted to test the compatibility of AtTFIIB1 in yeast transcription. Constructs designed to produce physiological levels or over-expression of AtTFIIB1 were transformed into yeast cells, and selection with 5-FOA forced the loss of plasmid-borne yTFIIB. No viable colonies were observed after plasmid substitution at either expression level.

Discussion

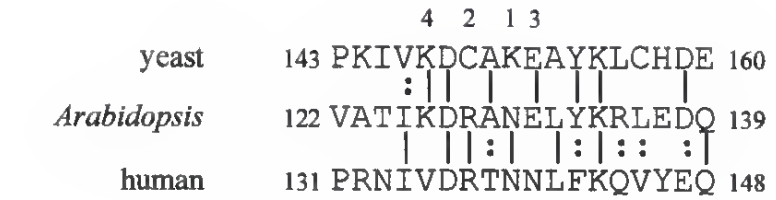
An accurate comparison of the AtTFIIB1 binding affinity for TBP from *Arabidopsis*, humans and yeast was difficult due to the variable EMSA conditions required to measure interactions and qualitative differences in the appearance of EMSA results. These differences also seemed to affect the more sensitive assays using fluorescence anisotropy (Figs. 3-5 and 3-6). AtTFIIB1 had high affinity for AtTBP and yTBP, but the yeast protein exhibited a different pattern of sensitivity to BSA. hTBP showed less relative binding to AtTFIIB1 under the standard assay conditions, but the interaction seems to be productive given the results from *in vitro* transcription. Different characteristics again appear in titrations of TBP-TATA binding. Addition of AtTFIIB1 in all cases gave greater anisotropy than when only TBP was present, but an influence on the apparent binding constant was only observed with AtTBP in Fig. 3-7. The inability to generate a normal binding curve with yTBP, even in the absence of TFIIB, indicates that alternative reaction conditions are needed to properly assay its activity as was the case for EMSA experiments. The yeast TFIIB protein may be susceptible to aggregation or precipitation since free probe disappeared without visible conversion to slower mobility complexes in the yTBP-AtTFIIB1 EMSA reactions. Although not seen in Fig. 3-2B, the

wells of yTBP EMSA gels occasionally showed significant amounts of radiolabeled probe that was apparently unable to enter the gel matrix.

The fluorescence polarization results suggest the nature of TFIIB species specificity is not strictly dependent upon the level of affinity for a TATA-TBP complex. Equilibrium anisotropy measurements predicted higher affinity binding of AtTFIIB1 to AtTBP and yTBP than to hTBP, yet the plant protein supported transcription from HeLa extracts but not in yeast substitutions. A comparison of the B1 helices from TFIIB homologs shows that the AtTFIIB1 sequence (and that of other plant homologs) is more conserved with human than yeast factors (Fig. 3-14), but AtTFIIB1 is equally conserved with hTFIIB and yTFIIB at the four residues within the helix that were initially implicated as being important for species specificity (Shaw *et al.*, 1996). Subsequent mutations of the four residues revealed that yeast K151 is most critical for *in vivo* function, followed by equal effects resulting from changing C149 or E152, and mutations at K147 were least disruptive (Shaw *et al.*, 1997). The identity between plant clones and hTFIIB at positions analogous to yeast amino acids 151 and 149 would therefore seem to be reflected in the inability of either TFIIB homolog to substitute into the yeast system. Helix B1 does not contact DNA or TBP in the ternary crystal structure (Nikolov *et al.*, 1995), so the interactions important for its function in yeast may occur at a subsequent step in PIC formation. Shaw *et al.* observed differential effects at various promoters for the yeast to human point mutations suggesting decreased ability to interact with some activators.

Fluorescence anisotropy was more useful for quantitation of AtTFIIB1 effects on the AtTBP binding curve. Models for PIC assembly start with the TBP-TAF complex

A.



B.

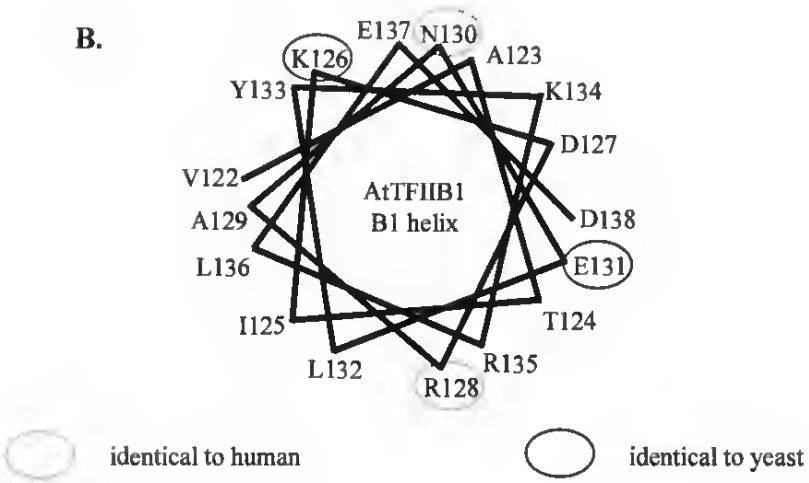


Figure 3-14. Comparison of the B1 α-helices from TFIIB homologs.
A. Alignment of B1 helix sequences shows plant clones are slightly more conserved with the human version than the yeast homolog. Residue positions are numbered at the ends of each sequence, and four residues critical for yeast plasmid substitutions are numbered in order of influence above the yeast sequence. **B.** A helical wheel projection of the B1 helix from *Arabidopsis* indicates conserved positions among the four critical residues do not map to the same face.

binding to the TATA region followed by interaction with TFIIB alone or as a part of a holoenzyme (Cujec *et al.*, 1997; Kim *et al.*, 1994; Koleske and Young, 1994). This order of assembly would predict TFIIB has little effect on the TBP k_a since TFIIB would not yet be present to influence the rate of association. This is supported by the observation that the decrease in the K_d of AtTBP for TATA mediated by AtTFIIB1 (Fig. 3-7) can be almost entirely accounted for by a 1.5-fold decrease in the AtTBP off-rate (Fig. 3-10). Curve fitting analysis was used to calculate the inflection points for binding assays in Fig. 3-7, and the results indicated the K_d of AtTBP alone for TATA was 34.1 nM. This value was reduced to 17.5 nM when AtTFIIB1 was present, a 1.9-fold increase in affinity. It is unclear why the observed AtTBP K_d for TATA was higher than previous measurements of 2-4 nM for yTBP and 5 nM for potato TBP (Hahn *et al.*, 1989; Holdsworth *et al.*, 1992; Hoopes *et al.*, 1992). The AdMLP probe used may not contain TATA or flanking sequences optimal for interaction with AtTBP, but TBP generally shows a fairly wide range of binding sequence specificity (Hahn *et al.*, 1989; Mukumoto *et al.*, 1993).

An initial estimate of the K_d for AtTFIIB1 binding to AtTBP-DNA is 7.1 nM. Attempts to confirm this value by independently measuring the k_a and k_d will require further optimization of the anisotropy assay or use of alternate detectors. The on-rate at various AtTFIIB1 concentrations was too fast to accurately observe at five-second intervals, and reducing the time of detection gave excessive increases in the margin of error. Measurement of the off-rate in this system requires high concentrations of competitor, which for AtTFIIB1 would be unlabeled and pre-formed DNA-AtTBP. While addition of large amounts of DNA oligomer did not affect fluorescence polarimetry, the presence of high protein concentrations can overwhelm the binding

signal with background fluorescence. Proper calculation of these kinetic rates will thus require more sensitive emission detectors which would allow the use of lower reactant concentrations, or switching to an immobilized ligand system such as those utilized by biosensor instruments (BIAcore or Affinity Sensors) to bypass the need for unlabeled competitor. The signal-to-noise problem may be one source of inaccuracy in the binding curves obtained for AtTFIIB1; in some cases the total fluorescence at high protein concentrations began to vary from the normal value (Figs. 3-8 and 3-12). Increased background fluorescence often prevented binding measurements at high reactant concentrations, and this translated to fewer observations near the upper asymptote of the binding plots. While curve fitting usually detected an upper plateau in the binding signal, a few more data points would help confirm the shape of these curves and their inflection points. It should also be noted that the AtTFIIB1 K_d is probably affected *in vivo* by the presence of TAFs that alter the DNA topology and TBP conformation (Burley and Roeder, 1996), and TAFs can provide additional interaction surfaces for TFIIB binding (Hori *et al.*, 1995).

CHAPTER 4
INTERACTION OF *ARABIDOPSIS* TRANSCRIPTION FACTOR IIB WITH TRANSCRIPTIONAL REGULATORS

Literature Review

Plant transcription regulatory factors contain many of the same classes of activation domains (AD) characterized in other eukaryotes. While a shared predominance of certain amino acids serves as the basis for such classification, it is becoming clear that not all members within these groups may activate transcription by the same mechanisms. Studies to make such distinctions, and identify mechanisms of activation in general, have progressed in human, yeast, and fruit fly systems, but have just begun in plants. This investigation will attempt to confirm predicted interactions between a plant pol II PIC component and two plant transcription factors, one of which contains an acidic AD and the other a proline-rich region.

Acidic activators are a large group of transcription factors containing a high proportion of negatively charged residues in their AD (Hope and Struhl, 1986; Ma and Ptashne, 1987). The AD of one of the archetypes, VP16, is composed of two adjacent subdomains with 25% acidic and only 4% basic residues. While overall charge is necessary but not sufficient for activation (Cress and Triezenberg, 1991), the bulky hydrophobic residues leucine, isoleucine and methionine that are interspersed within the acidic blocks are functionally critical (Regier *et al.*, 1993). Experiments using wild-type VP16 or fusions of its AD with DNA-binding domains (DBD) have demonstrated

regulation of transcription via TBP and TFIIB binding and PIC assembly (Ingles *et al.*, 1991; Roberts *et al.*, 1993; Stringer *et al.*, 1990), coactivator binding (Silverman *et al.*, 1994), enhanced elongation (Ghosh *et al.*, 1996), and anti-repressive histone or chromatin binding (Bunker and Kingston, 1996; Lyons and Chambon, 1995). VP16 mutants affecting *in vitro* binding and *in vivo* activation have implicated recruitment of TFIIB (Roberts *et al.*, 1995) as one mechanism of regulation, and also have been used to argue against TFIIB as a target (Gupta *et al.*, 1996). As a class, acidic activators show affinity *in vitro* for multiple members of the PIC and binding often occurs with both TBP and TFIIB, and sometimes to TFIIB but not to TBP (Chiang *et al.*, 1996; Haviv *et al.*, 1996; Tong *et al.*, 1995).

Several plant transcription factors have acidic ADs. Factors with homeodomain (Korfhage *et al.*, 1994), Ap2 (Stockinger *et al.*, 1997), and Myb (Urao *et al.*, 1996) homologies respond to various stress conditions and contain acidic activation regions. Proteins containing basic region/leucine zipper (bZIP) motifs, which direct DNA binding and factor dimerization, are often members of the acidic AD class (Unger *et al.*, 1993; Weisshaar *et al.*, 1991). Other examples include an embryo specific activator from bean, PvALF (Bobb *et al.*, 1995) and regulators of the maize anthocyanin pathway such as Lc (Ludwig *et al.*, 1989) and C1. Alignment of the C1 AD with VP16 shows similarity in amino acid sequences. The acidic C-terminus of C1 is localized to promoter elements through an N-terminal Myb DNA-binding domain, and was randomly mutagenized to determine what residues contribute to the activation function (Sainz *et al.*, 1997). A shared leucine is critical for both proteins, but changes at other hydrophobic positions important for VP16 had little effect on C1 in maize or yeast assays. An aspartate

mutation in C1 also decreased activity, but charge substitutions generally did not affect transcription. Whether these similarities and differences among acidic activators will be reflected in the details of their activation mechanisms is to be determined and will be aided by correlation of reduced activation potential with mutations that impair PIC interaction.

The *C1* gene promoter is regulated by another acidic activator, VIVIPAROUS1 (VP1), which also contains a repressor domain and affects transcription of seed developmental genes (Hoecker *et al.*, 1995; McCarty *et al.*, 1991). A cryptic C-terminal DNA-binding domain (Suzuki *et al.*, 1997) is adjacent to the negative regulatory region. The N-terminal AD contains three acidic blocks punctuated by bulky hydrophobic residues and a region rich in serine and threonine, another marker for some types of ADs (Triezenberg, 1995). This N-terminal pattern is also found in the dicot homolog of VP1, ABI3 from *Arabidopsis* (Parcy *et al.*, 1994). Experiments using an internal deletion construct and a gain-of-function hybrid fusion confirmed that two of the VP1 acidic domains, linked by the serine/threonine region, comprise a transcriptional AD in plant cells (McCarty *et al.*, 1991). Activity in yeast was not tested.

A second class of ADs is defined by enrichment for proline and was first observed in mammalian CTF/NF-1 and AP-2 (Mermod *et al.*, 1989). CTF and other proline activators have subsequently been shown to interact with TFIIB (Kim and Roeder, 1994; Malik and Karathanasis, 1996), and the CTF proline domain is organized in a manner similar to the CTD of the pol II largest subunit (Xiao *et al.*, 1994). Like the CTD, this protein was shown to bind to TBP in addition to TFIIB. The observed TBP interaction led to the proposal of an activation mechanism in which the proline domain displaces the

TBP-CTD interaction and thus disrupts a pol II link to the PIC, allowing the enzyme to progress to the elongation mode (Xiao *et al.*, 1994). Multiple PIC interactions are common for transcriptional activators, and many show functionally synergistic combinations of ADs such as TFE3 which contains both an acidic domain and a proline region (Artandi *et al.*, 1995).

A subgroup of the plant bZIP factors has putative ADs rich in proline. These include G-box binding factors (GBF) that recognize various forms of a consensus DNA element commonly found in activated and inducible plant promoters. *Arabidopsis* GBF1 was shown to direct transcription activation through its proline domain (Schindler *et al.*, 1992), and the high homology among GBFs suggests this function of the proline region is conserved. Soybean proteins SGBF1 and SGBF2 were isolated as binding factors for G-box related elements in a promoter regulated by auxin (Hong *et al.*, 1995). They exhibit typical GBF architecture including the N-terminal proline domain (19% P), a central nuclear localization sequence, and a C-terminal bZIP region. The soybean factors can heterodimerize, as can many leucine zipper proteins, and EMSA reactions containing SGBF1, SGBF2 or the two factors together show distinct DNA binding patterns in each case (Hong *et al.*, 1995). This plasticity in DNA binding specificity may have been exploited during evolution to generate promoter variants that selectively utilize GBF monomers, homo- or heterodimers. Dimer binding to promoter elements is another mechanism that could result in multiple ADs being present when needed for enhanced activation potential.

Screening for the interaction targets of ADs usually begins with affinity chromatography techniques to detect binding between the transcription activator and

components of the PIC. The interaction assay passes one protein reactant over a Sepharose resin column carrying immobilized fusion proteins of the second reactant, or combines the two in a low volume batch from which the resin beads can be recovered by slow centrifugation. Input reactant retained on the resin by interaction with the fusion protein is then eluted and detected, and the binding, wash and elution conditions can be manipulated to assess interaction affinities. Such *in vitro* assays allow rapid screening for binding and for effects of mutations that can be used to map the interaction domains. The results obtained help guide the design of *in vivo* experiments to confirm interactions, an important second step since *in vitro* binding events sometimes do not correlate with actual function (Tansey and Herr, 1995).

Two techniques have been developed to help improve such correlations. The yeast two-hybrid assay (Chien *et al.*, 1991; Fields and Song, 1989) fuses one potential reactant protein to a DBD which has specific affinity for a binding element in the promoter of a reporter gene, and the second reactant protein with a strong yeast AD. Interaction of the two proteins localizes the AD to the promoter and results in detectable transcription. Advantages of this system include *in vivo* conditions for interaction and eukaryotic protein expression and modification; of course, the latter may result in improper modification and the proteins of interest must be functionally stable as fusions with the yeast domains. A second improvement has been the development of very sensitive protein interaction detectors based on changes in refracted or reflected light. Surface plasmon resonance, for example, can be used to detect the interaction of a free ligand with a surface layer containing a second protein, and this event is measured in real time with continuous buffer flow (Jonsson *et al.*, 1991). The advantage over affinity

chromatography is an ability to quickly determine optimal binding conditions, view the results of variations in those conditions, and accurately discriminate between different ligands or mutants by measuring slight changes in binding kinetics. The technique has been used recently to measure affinities of PIC proteins for two acidic ADs, both of which interacted with TBP and TFIIB in previous affinity chromatography assays. Surface plasmon resonance indicated the human NF-κB subunit p65, which contains an acidic AD similar to VP16, bound TBP with two orders of magnitude greater affinity than the p65 interaction with TFIIB (Paal *et al.*, 1997). An acidic AD from yeast GAL4, however, showed only a 3-fold greater affinity for yeast TBP compared to TFIIB, and a series of AD mutants equally affected binding to both basal factors and displayed a linear range of binding constants that directly correlated to the level of activated transcription observed in yeast (Wu *et al.*, 1996).

The experiments described below represent an initial screen for interaction of AtTFIIB1 with the plant acidic activator VP1 and the proline-bZIP factor SGBF2. Limited success using the *in vivo* yeast two-hybrid assay was followed by affinity chromatography under a variety of conditions to probe interaction chemistry. Deletion constructs of AtTFIIB1 were also used to map regions of interaction.

Materials and Methods

Yeast Transcription and Two-Hybrid Assays

Yeast plasmids pGBT9 and pGAD424 (Clontech) were used to express fusion proteins from the constitutive promoter of *ADH1*. pGBT9 fuses the GAL4 DBD (amino acids 1-147) to the N-terminus of expressed proteins, and pGAD424 substitutes the

GAL4 AD (768-881) at the same location. PCR amplification was performed to attach *Eco*RI sites to a DNA fragment encoding the activation domain from VP16, amino acids 2076-2309, and this fragment was cloned into the *Eco*RI sites of both yeast plasmids. PCR amplification from pEThVP23 (Dr. Don McCarty, University of Florida) of the entire coding region for VP1 attached an *Eco*RI site before the first methionine and substituted an *Xho*I site for the stop codon using the primers 5VP1: 5' CCTGAATTCA-TGGAAGCCTCCTCCGGCT 3' and 3VP1: 5' TGCTCTCGAGGATGCTCACCGCC-ATCTG 3'. The resulting fragment was ligated to the *Eco*RI and *Sal*I sites of the yeast vectors. Deletions of VP1 were constructed in the yeast plasmids using an in-frame *Bam*HI site at residue 191. An *Eco*RI-*Bam*HI digestion of the PCR product and ligation into those sites in the yeast vectors produced the construct VP1a encoding the N-terminal one-third of VP1. A *Bam*HI-*Pst*I fragment from the PCR product encodes the central portion from residues 192-406 and was ligated into those sites for yeast expression of VP1b. The C-terminal region (VP1c, residues 413-691) is encoded on a *Pvu*II fragment of the amplification product and was blunt-end ligated into the *Sma*I site of pUC19. Digestion of that construct with *Eco*RI and *Xho*I released the VP1c fragment for ligation into the yeast plasmids at the *Eco*RI and *Sal*I sites. The plasmid pAtTFIIB1 (Chapter 3) was digested with *Kpn*2I and *Sal*I, and the resulting fragment was cloned into the *Psp*A1 and *Sal*I sites of pGBT9.

Activation of transcription in yeast strain PCY2 was measured by assaying β-galactosidase (β-gal) activity that results from expression of a chromosomal *lacZ* gene under control of a promoter containing GAL4 binding sites. Yeast cells transformed with one or both of the expression vectors were plated on the appropriate auxotrophic selection

media, and five independently transformed colonies were grown for liquid culture β-gal assays as described (Miller, 1972). Cell extracts were incubated with o-nitrophenyl β-D-galactopyranoside for 20 min at 30° C before quenching.

In vitro Interaction Assays

pGEX vectors designed for bacterial expression of proteins fused at the N-terminus to glutathione S-transferase (GST) were used to create immobilized protein affinity resins. The *Eco*RI fragment from pEThVP23 was ligated into pGEX-2TK (Pharmacia) to produce pGEXVP1, and the *Bam*H1 fragment from pGEXVP1 was re-cloned into pGEX-2TK to create pGEXVP1a. Primers incorporating a *Bam*H1 site at the 5' end and an *Eco*RI site at the 3' end of a cDNA for SGBF-2 (Dr. Tom Guilfoyle, University of Missouri) were used to PCR amplify the entire coding region and clone it into pGEX-KG. The primers used were 5GmGBF2: 5' GCTGGATCCATGGGAAA-CAGTGAGGAAGAG 3' and 3GmGBF2: 5' ACTGAATTCAACCAGCTGCTACA-GCATTAG 3'. A PCR reaction was also used to attach *Bam*H1 and *Eco*RI sites to the VP16 AD for cloning into pGEX-2TK. Stationary phase *E. coli* BL21 cultures transformed with pGEXVP1a, pGEXSGBF2, or pGEXVP16 were diluted 1:200 and incubated 2.5 hr at 37° C before induction with 1 mM IPTG. Incubation continued for 3 hr and then cells were pelleted, resuspended in 1X PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) with protease inhibitors (Chapter 3) and 1% Triton X-100, and sonicated. Cleared lysates were batch purified by binding to glutathione-Sepharose resin (GS4b, Pharmacia) and washed with 1X PBS.

Deletion mutants of *AtTFIIB1* were generated by PCR amplification of the cDNA using primers that incorporated appropriate restriction sites. Amplified regions were

cloned into pET24b for HisBind purification and Western detection of the T7 epitope fused to the N-terminus. Purification of intact peptides was achieved for constructs carrying several AtTFIIB1 domains, including residues 1-215 (Zn ribbon to repeat 1, ΔR2), 44-312 (conserved region to repeat 2, ΔZn), 100-312 (repeats 1 and 2, core), and 100-215 (repeat 1, R1). Expression and purification of these proteins and full length AtTFIIB1 were as described in Chapter 3.

GS4b-immobilized VP16, VP1a, SGBF2, or GST alone (10-15 μg total protein per assay) was mixed with an equal packed volume of unbound beads to create an affinity resin mixture suitable for retention of GST fusion proteins through multiple manipulations. Resin batches were collected by low speed centrifugation into a packed volume generally between 20-30 μl, and then mixed with 1-2 μg AtTFIIB1 in BS buffer without glycerol (Chapter 3) in a final volume of 60 μl. Binding reactions were gently rocked at room temperature for 30 min with occasional additional mixing. Supernatant fractions collected after binding included flow-through (reactants not bound to resin), three washes (each with 3X bed volume of interaction buffer), three elutions (3X bed volume of buffer with various salt and/or detergent concentrations), and bound (reactants released into buffer by boiling the resin). When multiple elutions were tested in one binding assay, each sequential step was comprised of three elution fractions per treatment. Detergents used for elution above and below their critical micelle concentrations (CMC) included sodium dodecyl sulfate (SDS, CMC 7-10 mM), cetyltrimethylammonium bromide (CTAB, CMC 1 mM), and TRITON X-100 (TX100, CMC 0.29 mM).

Western Blot Detection of AtTFIIB1

One-third of the total volume from each protein fraction was electrophoresed on 12% SDS-polyacrylamide (30:1) gels and transferred to PVDF membranes (Immobilon-P, Millipore) using a Trans-Blot SD electrophoretic cell (Bio-Rad) according to the manufacturer's instructions. Blots were washed and incubated in TBST (0.1 M Tris-HCl pH 7.5, 0.9% NaCl, 0.1% Tween-20) with a 1:10,000 dilution of anti-T7 mouse antibody (Novagen) for 1 hr at room temperature. TBST washes to remove the primary antibody were followed by incubation with a 1:10,000 dilution of anti-mouse IgG antibody conjugated with horseradish peroxidase (Promega) for 45 min at room temperature. Chemiluminescent detection was performed with the ECL protocol (Amersham) and blots were exposed to Kodak XR-Blue X-ray film for 15, 30 and 60 sec. Detected bands were quantified by densitometry with the IS1000 system.

Results

In preparation for two-hybrid interaction screening, fusion constructs of GAL4 DBD-VP1 were transformed into yeast to determine whether the maize factor was properly expressed and functionally active. As shown in Table 4-1, full length VP1 activated transcription of the *lacZ* reporter. The transcriptional activity maps to the N-terminal 191 amino acids (VP1a), and the level of activity is comparable to that from the positive control, VP16. Cultures transformed with the remaining VP1 regions, VP1b and VP1c, produced no detectable β-gal. The ADs of VP1 and VP16 were then transferred to the GAL4 AD fusion vector and co-transformed with AtTFIIB1 fused to the GAL4 DBD. The negative control containing AtTFIIB1 alone gave no transcription (Table 4-1), but

neither did the putative positive control co-expressing VP16, which is known to interact with TFIIB in *in vitro* systems (Roberts *et al.*, 1993). VP1a+AtTFIIB1 also showed no detectable β-gal activity.

Table 4-1. Activation of yeast transcription by fusion proteins containing the GAL4 DBD or the GAL4 AD. Average reporter activity is indicated with standard deviation (SD).

GAL4 fusions: DBD-	AD-	β-gal (Miller)	+/-SD
VP16		1.33	0.33
VP1		0.79	0.09
VP1a		1.16	0.14
VP1b		0	
VP1c		0	
AtTFIIB1		0	
AtTFIIB1	VP16	0	
AtTFIIB1	VP1a	0	

AtTFIIB1 was next tested in an *in vitro* interaction assay. GST fusions with the ADs of VP16 and VP1, and the entire SGBF2 protein, were expressed in *E. coli* and immobilized on GS4b resin. AtTFIIB1 was also *E. coli* expressed, purified via the C-terminal His tag, and detected in binding reactions by Western blotting using antibodies against the N-terminal T7 epitope. This AtTFIIB1 configuration with short peptide tags on each terminus was previously shown to be functionally active (Chapter 3). Incubation of AtTFIIB1 with immobilized VP16, VP1a, or SGBF2 generally resulted in retention of about 40% of the input protein, and the binding was specific for the ADs since no AtTFIIB1 was retained on resin carrying only GST (Fig. 4-1). The interaction with all

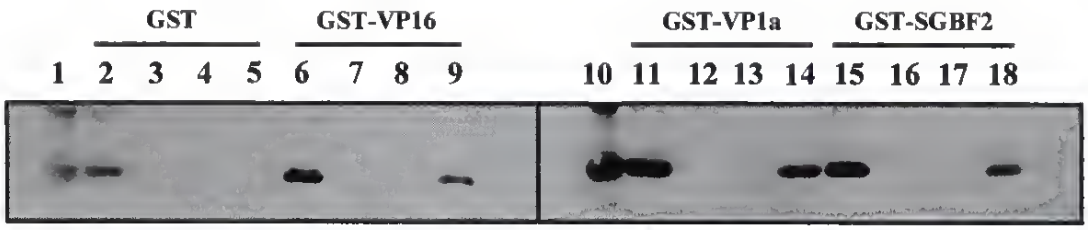


Figure 4-1. Western blot detection of AtTFIIB1 from affinity chromatography fractions. AtTFIIB1 was incubated at room temp. with immobilized fusion proteins in buffer containing 50 mM KCl. After collecting the flow-through fraction and three washes in binding buffer, three elution fractions were collected for each sequential KCl treatment described. Protein that remained associated with the resin was released by boiling. Trace amounts of AtTFIIB1 were detected in the first wash fractions; only the flow-through and first elution fractions are shown. One-third of the total fraction volume was loaded on the gel for blotting. Lanes 1 and 10, 10% input AtTFIIB1; lanes 2, 6, 11 and 15, flow-through; lanes 3, 7, 12 and 16, elution with 0.5 M KCl; lanes 4, 8, 13 and 17, elution with 1.0 M KCl; lanes 5, 9, 14 and 18, boiled. The immobilized protein present in each reaction is indicated over the lane numbers.

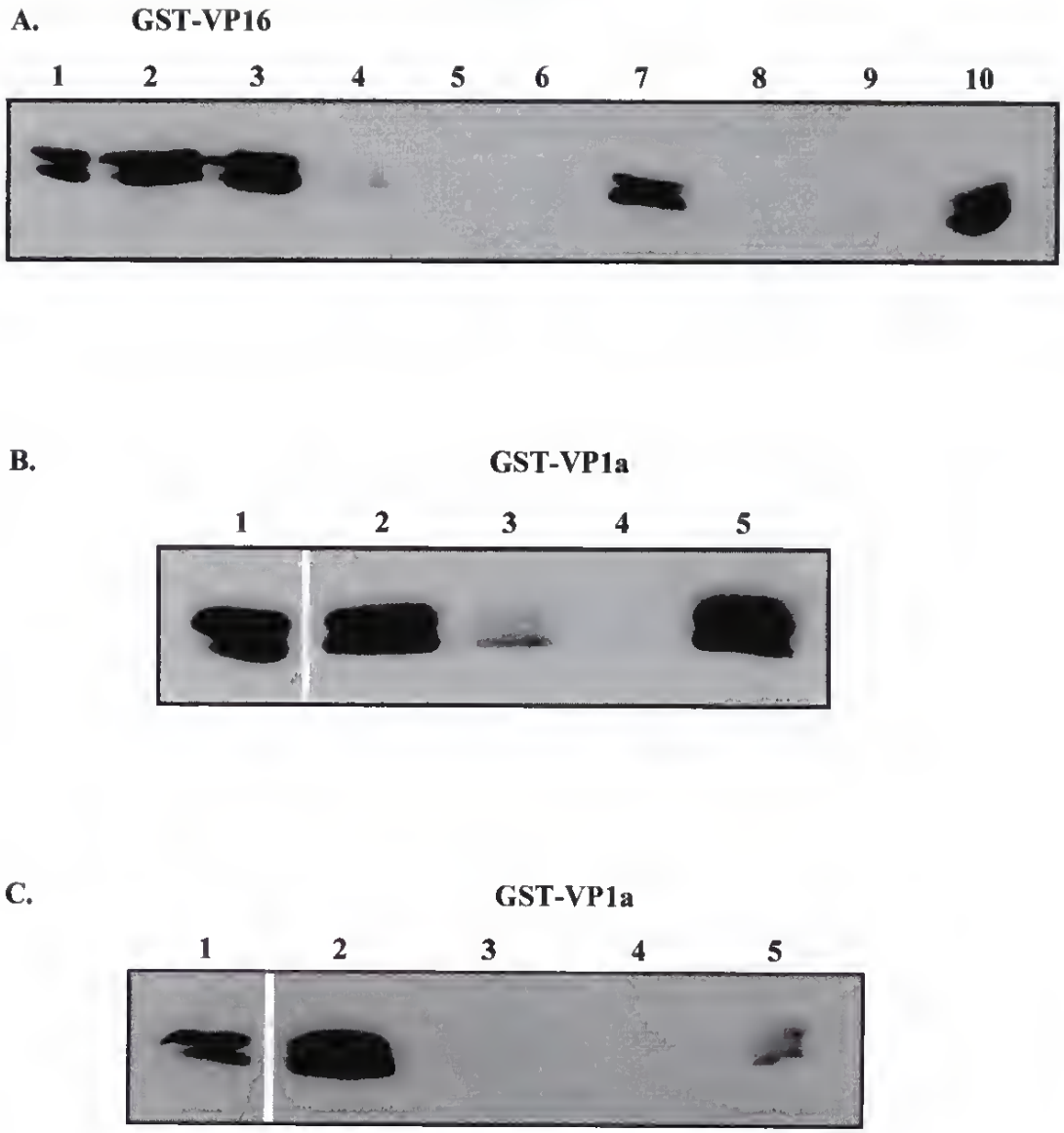


Figure 4-2. Ethylene glycol elution of AtTFIIB1 from acidic activation domains. **A.** AtTFIIB1 (10% input, lane 1) is detected in flow-through (lane 2) and bound (lane 3) fractions after interaction with immobilized VP16. A replicate reaction was sequentially eluted with 10% (lane 4), 30% (lane 5), and 50% (lane 6) ethylene glycol in 0.1 M KCl, but most AtTFIIB1 remains bound (lane 7). A third reaction was eluted with 30% ethylene glycol in 0.5 M (lane 8) or 1.0 M (lane 9) KCl, lane 10 is the boiled fraction. **B.** AtTFIIB1 (10% input, lane 1) fractions after interaction with VP1a include flow-through (lane 2), sequential elution with 10% (lane 3) or 50% (lane 4) ethylene glycol in 0.1 M KCl, and boiled (lane 5). **C.** Results of a duplicate reaction as in B., except elution buffer was supplemented with 1.0 M KCl.

three ADs was stable in high salt concentrations since no AtTFIIB1 was detected in elution fractions containing 0.5 M or 1.0 M KCl.

TFIIB binding to the initiator-binding factor YY1 has also been recently shown to be very salt-stable (Usheva and Shenk, 1996), but TFIIB could be released using an elution buffer with 50% ethylene glycol, which is known to disrupt hydrophobic interactions (Thompson *et al.*, 1990). Ten percent ethylene glycol released a slight fraction of the AtTFIIB1 bound to VP16, and additional sequential elutions with 30% or 50% ethylene glycol had no effect (Fig. 4-2A). High KCl concentrations combined with ethylene glycol were ineffective, and the same results were obtained with AtTFIIB1 binding to VP1a (Fig. 4-2B and C). To further investigate the susceptibility of AtTFIIB1 interactions to disruption, three detergents were included in the elution buffers. Each detergent was tested at concentrations below and above the point at which monomeric molecules aggregate to form micelles, or the critical micelle concentration (CMC) (Neugebauer, 1994). As shown in Fig. 4-3A and Fig. 4-4, the anionic detergent SDS completely eluted AtTFIIB1 from immobilized VP16 and VP1a while nearly none was released by the cationic detergent CTAB. A non-ionic reagent, TX100, eluted a portion of the AtTFIIB1 with each increase in detergent concentration but did not completely disrupt binding to VP16 or VP1a. The contrasting abilities of CTAB and SDS to affect binding were also seen upon comparison at equimolar concentrations (Fig. 4-3B).

Binding of AtTFIIB1 to SGBF2 showed similar results as with VP16 and VP1a. SDS again completely eluted the bound AtTFIIB1, but CTAB was slightly more effective for disrupting this interaction compared to the acidic ADs (Fig. 4-5). Detectable AtTFIIB1 was found in elution fractions at both CTAB concentrations, but the majority

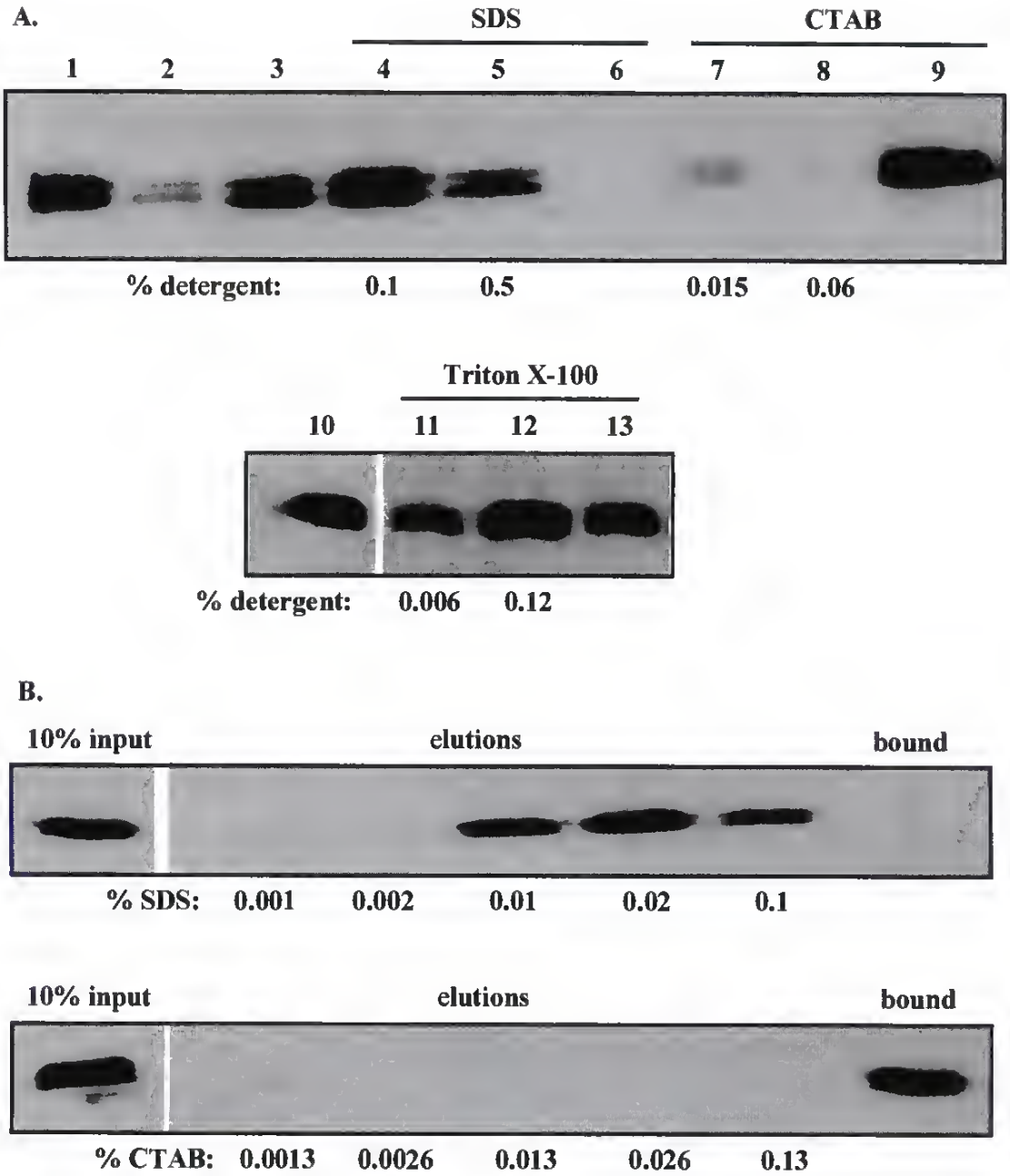


Figure 4-3. Detergent elution of AtTFIIB1 from immobilized VP16.
A. Lanes 1 and 10, 10% AtTFIIB1 input; lane 2, bound to immobilized GST (negative control); lane 3, bound to GST-VP16 (positive control). Replicate VP16 interactions were sequentially eluted by detergents at 0.5X (lanes 4, 7 and 11) and 2X (lanes 5, 8 and 12) their cmc, and then boiled to release any AtTFIIB1 that remained bound (lanes 6, 9 and 13). **B.** Progressive increases at equivalent molar concentrations of SDS and CTAB do not show the same elution patterns in parallel AtTFIIB1-VP16 interactions.

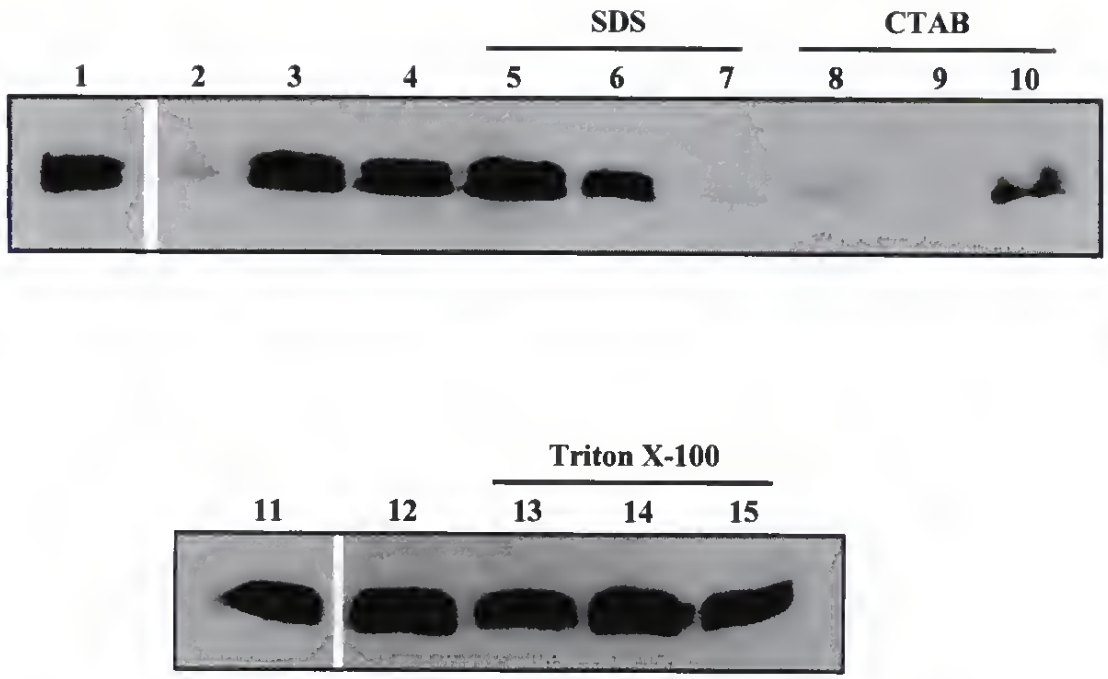


Figure 4-4. Detergent elution of AtTFIIB1 from immobilized VP1a.
Lanes 1 and 11, 10% AtTFIIB1 input; lane 2, bound fraction after interaction with immobilized GST (negative control); lane 3, flow-through; lanes 4 and 12, bound after GST-VP1a interaction (positive control). VP1a interactions with AtTFIIB1 were disrupted by elution with detergents at 0.5X (lanes 5, 8 and 13) and 2X (lanes 6, 9 and 14) their cmc, and AtTFIIB1 that remained in the bound fraction is shown (lanes 7, 10 and 15). Detergent concentrations were sequentially increased and are the same as Fig. 4-3A.

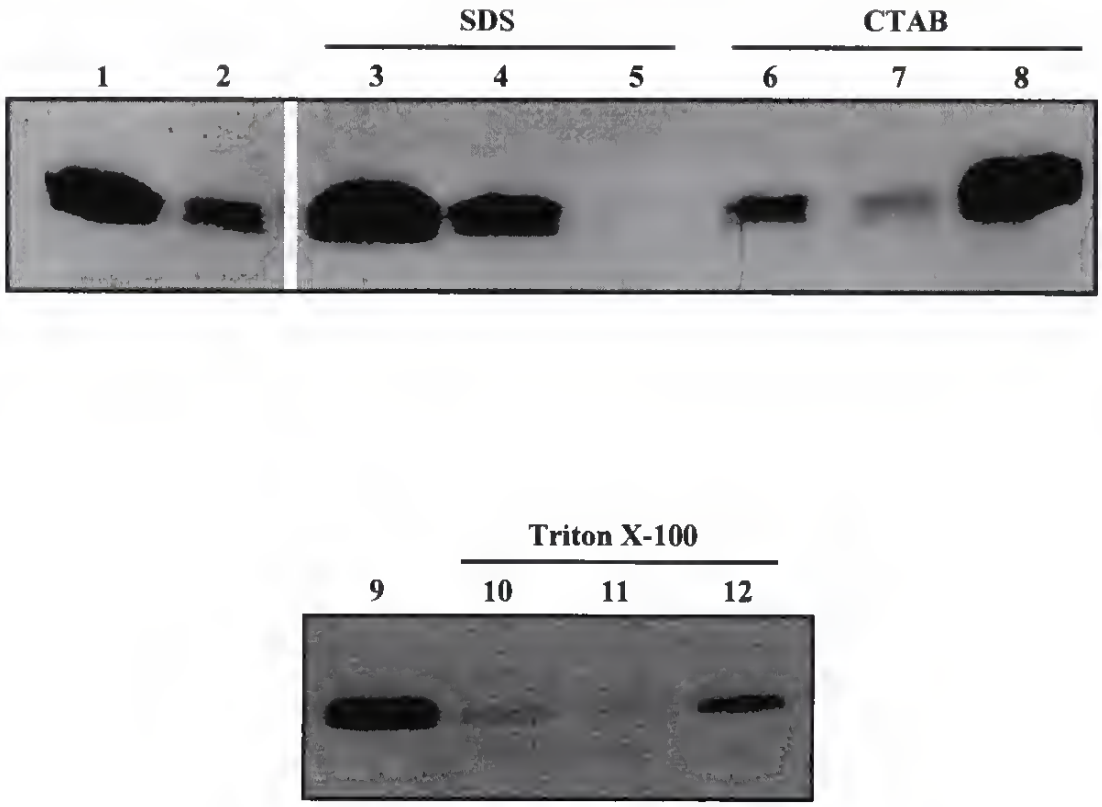


Figure 4-5. Detergent elution of AtTFIIB1 from immobilized SGBF2.
Lanes 1 and 9, 10% AtTFIIB1 input; lane 2, bound fraction after interaction with immobilized GST (negative control). SGBF2 interactions with AtTFIIB1 were disrupted by detergents at 0.5X (lanes 3, 6 and 10) and 2X (lanes 4, 7 and 11) their cmc, and AtTFIIB1 that remained in the bound fraction is shown (lanes 5, 8 and 12). Blots are overexposed to visualize low protein concentrations in lanes 7, 10 and 11.

remained associated with SGBF2. TX100 was less able to affect the interaction and gave slight elution of AtTFIIB1, with most remaining in the bound fraction. The results for each of the binding reactions with VP16, VP1a and SGBF2 were quantified by densitometry and combined with data from other binding experiments not shown. The amount of AtTFIIB1 detected in the flow-through and elution+bound fractions was calculated as a percentage of the input AtTFIIB1, and the results are listed in Table 4-2. Generally, the two acidic ADs retained AtTFIIB1 at a level 35% greater than the GST background, and SGBF2 bound 44% over GST alone.

Table 4-2. Proportion of AtTFIIB1 retained by *in vitro* interaction with transcription activators. Average protein detected relative to input is indicated with standard deviations (SD).

	% AtTFIIB1 in chromatography fraction:			
Immobilized	flow-through	SD	elution+bound	SD
GST	87.1	11.6	8.7	4.9
GST-VP16	40.6	13.9	42.3	12.7
GST-VP1a	47.9	18.1	46.9	16.4
GST-SGBF2	41.0	12.4	53.3	21.3

Affinity chromatography was also used to identify domains within AtTFIIB1 that interact with VP1 and SGBF2. Deletion constructs of AtTFIIB1 that were stably expressed in *E. coli* included a removal of the Zn ribbon (ΔZn), deletion of the second repeat (ΔR2), both repeats only (core), and repeat 1 alone (R1) (Fig. 4-6A). As shown in Fig. 4-6B, ΔZn retained the ability to interact with both VP1a and SGBF2 and did not bind to the negative control, GST. The core domain also retained interaction ability but with reduced affinity for VP1a compared to SGBF2 (Fig. 4-7A). ΔR2 appeared to bind

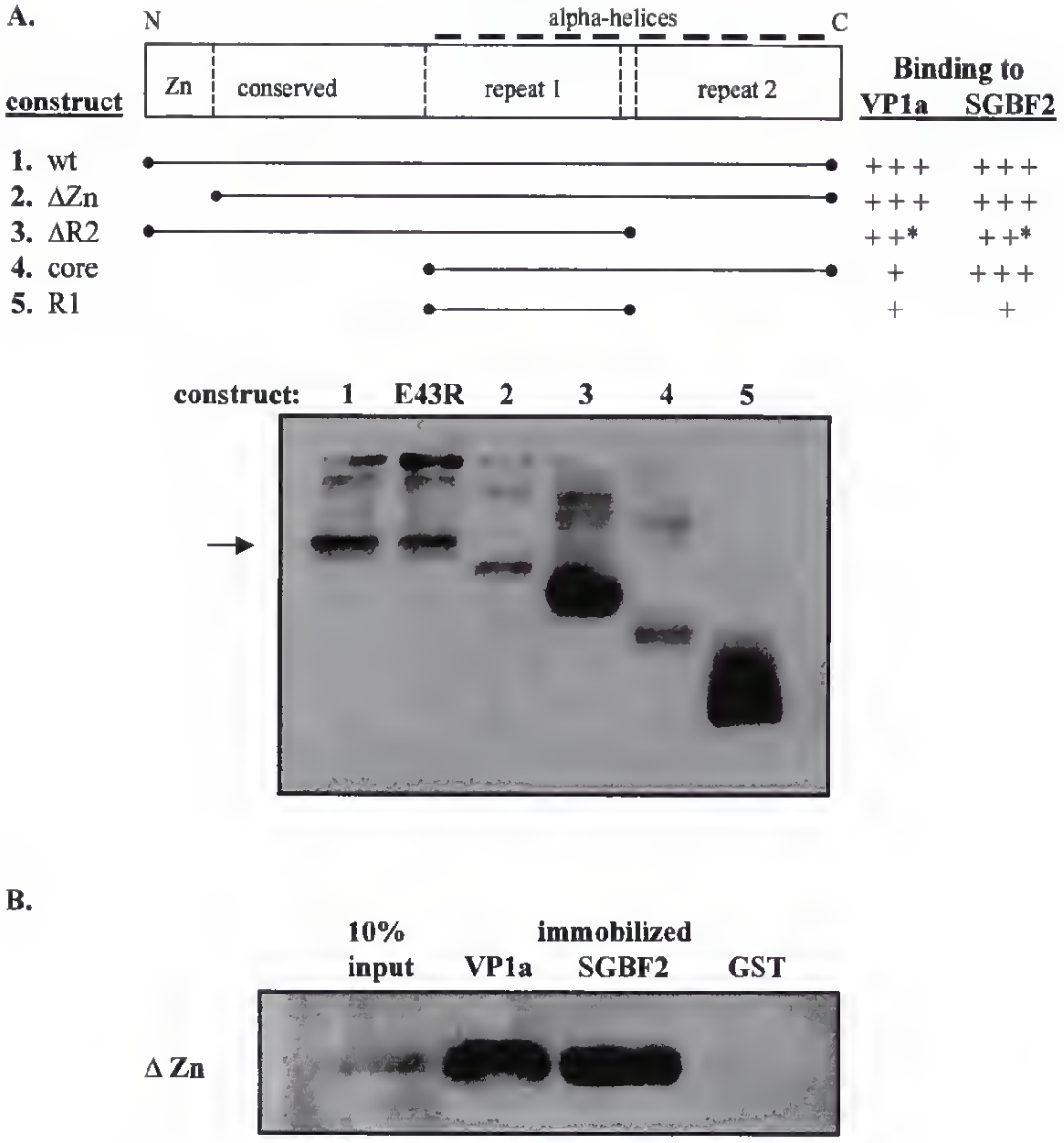


Figure 4-6. AtTFIIB1 deletion constructs for mapping interaction domains.
A. Five AtTFIIB1 constructs with termini shown in the diagram were tested for ability to interact with immobilized VP1a and SGBF2. Binding relative to wild-type AtTFIIB1 is indicated; asterisks denote significant interaction with the GST negative control. Western blotting using anti-T7 epitope antibody detects the AtTFIIB1 deletion constructs as well as protein from the AtTFIIB1 mutant E43R (Chapter 3). The arrow indicates full-length AtTFIIB1. **B.** The AtTFIIB1 construct ΔZn was added to binding reactions containing immobilized GST-VP1a, GST-SGBF2, or GST alone. After multiple washes in the binding buffer (50 mM KCl), the resin was boiled to release retained protein. A Western blot of the resulting boiled fractions is shown from the indicated reactions, adjacent to a lane containing 10% of the input AtTFIIB1 ΔZn.

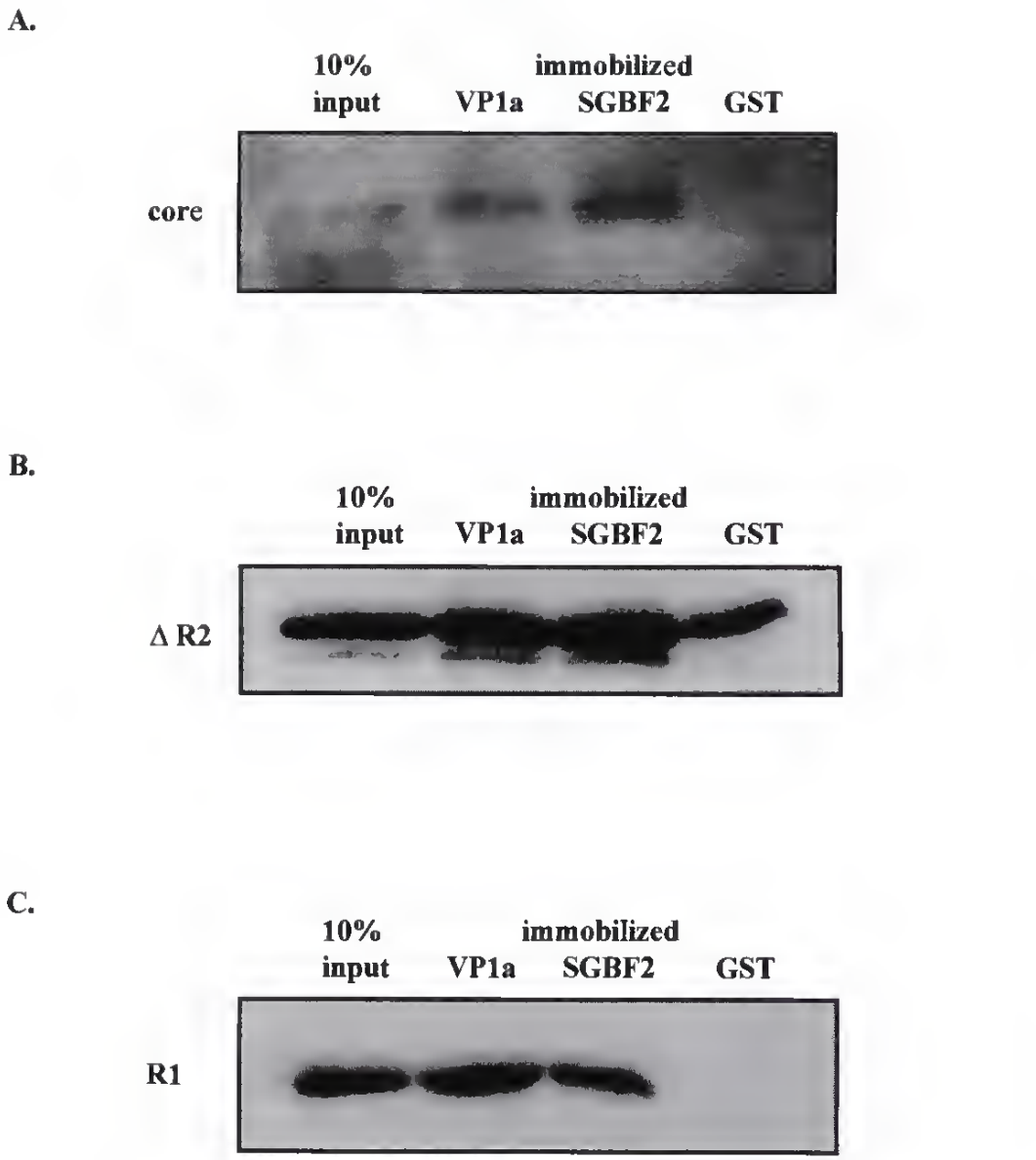


Figure 4-7. Mapping of AtTFIIB1 interaction domains with deletion constructs. All reactions were performed as described in Fig. 4-6B. Bound fractions were analyzed after boiling to release AtTFIIB1 constructs containing the core (**A.**), ΔR2 (**B.**), or R1 alone (**C.**).

both activators, but the specificity of this interaction is questionable since the peptide also had affinity for the GST protein alone (Fig. 4-7B). Finally, the R1 region interacted in Fig. 4-7C to an equal degree with VP1a and SGBF2, which both retained about 10% of the total input protein. This level of affinity is reduced compared to the wild-type (Table 4-2) or ΔZn constructs.

Discussion

As predicted from results in other eukaryotic systems, a plant TFIIB homolog interacts *in vitro* with a plant acidic AD and a transcription factor containing a proline-rich domain. Given the large number of transcription factors that show *in vitro* affinity for TFIIB, it will be interesting to determine whether correlations exist between the class of activation domain involved, *in vitro* binding characteristics such as the salt elution profile, and *in vivo* activation mechanisms. The highly stable interactions of TFIIB in moderate to high salt concentrations has been observed previously (Agostini *et al.*, 1996; Chiang *et al.*, 1996; Usheva and Shenk, 1996) but is not always the case; c-Jun binding to TFIIB, for example, is abolished between 0.2 and 0.3 M NaCl, while binding to TBP can be detected at 1.0 M NaCl (Franklin *et al.*, 1995). Other *in vitro* characteristics that may be useful for differentiating among TFIIB interactions include elution by hydrophobic reagents such as ethylene glycol, which disrupted binding with YY1 (Usheva and Shenk, 1996) but not with the VP1a acidic region, and elution by detergents. CTAB appears to release AtTFIIB1 from SGBF2 to a greater extent than from VP1a, but the opposite pattern was seen for TRITON X-100. In contrast to CTAB and despite an overall structural similarity, SDS consistently eluted AtTFIIB1 from all interactions tested. The

anionic SDS may be better suited for close contacts with AtTFIIB1 if basic residues are more often adjacent to the hydrophobic regions of interaction. Alternatively, this result may simply reflect variable strengths among detergents for general protein denaturation rather than specific interference at interacting domains.

Establishing specificity and relevance for any of these effects requires *in vivo* testing. The yeast two-hybrid assay has proven very useful for screening interactions under physiological conditions, but AtTFIIB1 failed to function in this system. Only two successful assays of TFIIB in the two-hybrid approach have been reported, and one of these studies fused hTFIIB to the GAL4 AD rather than to the DBD (MacDonald *et al.*, 1995; Masuyama *et al.*, 1997) to map its binding domain for the vitamin D receptor. A second approach detected interaction of the NF-κB subunit p65 with hTFIIB attached to the LexA DBD (Schmitz *et al.*, 1995). AtTFIIB1 may be improperly modified during expression in yeast, and this could explain why it failed to replace yTFIIB in plasmid shuffle experiments (Chapter 3). However, hTFIIB also does not substitute in yeast, yet interacts with the vitamin D receptor and p65 in the two-hybrid system. Another possibility is that fusion with the GAL4 DBD creates a non-functional conformational change in AtTFIIB1, or initiates assembly of a non-functional or improperly positioned PIC. This type of fusion is not detrimental to TBP and has been used to demonstrate activation of transcription by tethering the factor to promoters *in vivo* (Chatterjee and Struhl, 1995; Klages and Strubin, 1995; Xiao *et al.*, 1995), but there are no reports using TFIIB in such an experiment. Other promising *in vivo* techniques include the squelch and rescue system (Colgàn *et al.*, 1995) in which mutant TFIIB binds a co-expressed activator and represses transcription by preventing further PIC assembly. The squelch is then

relieved by introducing additional TFIIB point mutations that prevent activator binding. An altered specificity system might also be developed to create combinations of activators and PIC components that are only functional when co-expressed (Tansey and Herr, 1997), thus avoiding interference by endogenous factors or affecting basal transcription in a general manner, which must be accounted for with proper controls in the squelch assay.

These and other approaches will rely on *in vitro* binding results to map interaction domains and target regions for further study. This type of mapping for AtTFIIB1 interaction with VP1a suggests the same domains are involved as were observed for the VP16 acidic AD. Deletion mutations in both hTFIIB repeats affected VP16 binding, and point mutations in the E1 helix altered residues critical for interaction (Roberts *et al.*, 1993). AtTFIIB1 binding to VP1a was unaffected by removal of the Zn ribbon domain but a further N-terminal deletion to the first repeat resulted in retention of only 10% of the input protein (ΔZn vs. core, Figs. 4-6B and 4-7A). Unlike hTFIIB with VP16, the conserved domain of AtTFIIB1 apparently makes a significant contribution to interactions with VP1a. Binding reactions with AtTFIIB1 repeat 1 alone retained a similar amount of protein compared to the core, suggesting this region and its E1 helix are important for VP1a binding as was observed for VP16. The relative contribution of repeat 2 was difficult to judge and will require further study with constructs capable of maintaining solubility during purification while avoiding non-specific binding affinity for GST.

Interaction mapping for AtTFIIB1 with SGBF2 produced results similar to those for VP1a, but no reductions in affinity were observed upon deletion of the Zn ribbon or

conserved domains. Less protein was retained with repeat 1 alone compared to the core region (Fig. 4-7A vs. C), suggesting the presence of SGBF2 interaction surfaces in both repeats 1 and 2 of AtTFIIB1.

CHAPTER 5
CONCLUSION

The lack of reliable *in vitro* transcription systems from plants has slowed progress in characterizing the many regulatory factors known to influence plant gene expression. Identification of amenable cell cultures and improvements in protein extract preparation have begun to offer viable options for plant *in vitro* transcription (Zhu, 1996), and the rapidly growing databases of plant expressed sequence tags may soon provide another alternative. As more PIC component homologs are cloned, the fastest route to improved transcription systems may be the combination of well-purified RNA polymerase II or holoenzyme with selected recombinant basal factors. Extra purification of pol II avoids introduction of problematic inhibitory components and addition of appropriate *E. coli* expressed PIC factors should restore the capability for basal transcription. The capacity for regulation by upstream factors may require additional proteins that could be characterized from chromatographic fractions or identified by adding new PIC members as they are cloned. The starting point, however, is the minimum combination of factors needed to produce a basal level of transcription. For mammalian *in vitro* transcription, this minimum combination generally contains recombinant TBP, TFIIB, TFIIF, purified fractions of TFIIE/H, pol II, and sometimes TFIIA (Maldonado *et al.*, 1990). Characterizations of plant TBP, pol II, and now TFIIB represent necessary steps toward reconstituting a transcription system, and the pace of this progress will increase as factor cloning relies less upon inter-species homology to generate probes for screening cDNA

libraries and, instead, can start with randomly isolated cDNA tags from large scale genome projects.

The isolation of TFIIB from *Arabidopsis* is an example of the potential benefits of such complementation between library screening and cDNA tag cataloguing. Probe hybridization with nearly one million plaques from the *Arabidopsis* cDNA library did not recover a clone for *AtTFIIB2* and sequencing of expressed tags did not produce an isolate for *AtTFIIB1*, despite the use of the same ecotype for both approaches. Prior availability of *AtTFIIB2*, though, might have greatly increased the speed with which clones could be recovered from cDNA libraries and this will hopefully be true for other plant PIC members. Screening nearly two million plaques from the first soybean cDNA library produced only partial sequences for *GmTFIIB*, but the first round of plaque lifts using the second library recovered the full-length clone. It is therefore uncertain whether sufficient screening of a high quality library has been conducted to isolate other potential copies of the gene. Gene duplication of basal factors is, so far, unique to plants and was first observed for TBP from *Arabidopsis*, maize and wheat and now includes TFIIB from *Arabidopsis* and is suspected in maize. The very high homology between TBP homologs from individual species is reflected to a lesser degree between *AtTFIIB1* and *AtTFIIB2*, so it would be interesting to investigate whether gene copies for either TBP or TFIIB have functional differences (Heard *et al.*, 1993; Vogel *et al.*, 1993) or are just the result of genomic redundancy often observed in plants. Experiments addressing this question for TFIIB in *Arabidopsis* could include isolation of each gene's promoter to characterize expression patterns combined with TFIIB gene-specific Northern blots using RNA from various tissues and developmental stages. *In vitro* binding reactions with each of the four

combinations of TFIIB and TBP could also be conducted to detect any differences in affinity.

Initial characterizations of AtTFIIB1 indicate three functional similarities to hTFIIB. First, the plant factor substitutes for hTFIIB in an *in vitro* transcription assay using HeLa nuclear extract. The cytomegalovirus (CMV) promoter driving this transcription contains several upstream regulatory elements, including CAAT boxes and Sp1 sites, and detected transcripts are considered to be the result of activated transcription (Batt, 1996). AtTFIIB1 would thus seem to not only function in the context of the mammalian basal PIC, but may also be compatible with at least some of the various activators influencing the CMV promoter. Second, AtTFIIB1 and hTFIIB are both unable to substitute for the yeast homolog *in vivo*. This finding is consistent with comparisons of the TFIIB helix B1 in which changes at either of the two most important positions converted yTFIIB residues to those found in hTFIIB and AtTFIIB1 and severely affected yeast cell viability (Shaw *et al.*, 1997). This effect appears to be due to a lack of activated transcription from a subset of yeast promoters (Shaw *et al.*, 1996), suggesting that differences in activator binding affinities or functional mechanisms may play a role in TFIIB species specificity. Other differences between yeast and human systems may include holoenzyme composition, the role of TAFs, and promoter architecture. The degree to which plants functionally conserve these and other factors with yeast versus animals is not known. The results described for AtTFIIB1 would seem to show more compatibility with the HeLa system, but confirmation would require additional detailed assays such as *in vivo* activity of AtTFIIB1 and yTFIIB at basal and activated levels in HeLa and perhaps substitution experiments for hTFIIB and yTFIIB into plants. Certainly

eukaryotic transcription shows many more overall similarities than differences among species, and data on compatibility or specificity will probably be accumulated as a secondary priority to understanding regulation mechanisms.

A third similarity between AtTFIIB1 and hTFIIB, and other TFIIB homologs, is the ability to interact *in vitro* with the acidic domains of VP16 and with a proline-rich bZIP protein. Large scale deletion mapping of AtTFIIB1 interactions with the VP1 acidic AD is consistent with similar experiments for VP16 that indicated a primary binding domain in the first repeat. The functional significance of the TFIIB-acidic AD interaction is receiving much attention, and the VP1a construct may be a useful tool for studying activated transcription in plants. The relative affinity of VP1a for TBP should first be assessed, as well as affinity for any other PIC components available, to determine how many potential interaction targets exist. Alanine scans or random mutagenesis approaches are usually applied next to screen for individual residues in the activator important for *in vitro* binding, and mutants with reduced affinity are tested *in vivo* for reduced activation potential. Applying the same strategy to AtTFIIB1 could produce a similar map for its binding surfaces, but greater care must be taken to control for non-activator-specific effects such as reduced TBP or DNA binding. In plants, such a squelch and rescue study might utilize the CaMV 35S promoter deleted to around –90 with upstream GAL4 binding sites attached. This reporter would produce a basal level of transcription in the absence of an activator such as the GAL4 DBD-VP1a, and basal transcription should remain unaffected by expression of AtTFIIB1 mutants being screened for activator interactions. Characterization of SGBF2 affinity for AtTFIIB1 would also benefit from this strategy but should be preceded by localization of the

interaction domain(s) within the soybean protein. While the proline region is the most likely binding domain, the bZIP portion of c-Jun has been shown to bind through the E1 and E2 helices of hTFIIB, and both core repeats containing these helices were also important for the SGBF2-AtTFIIB1 interaction. *In vivo* experiments indicated that the bZIP region of c-Jun contains an AD and that TFIIB binding is dependent upon dimerization of the Jun protein (Franklin *et al.*, 1995). The potential dimerization of both soybean GBFs should be investigated for effects on *in vitro* interactions with AtTFIIB1, followed by an *in vivo* assay of SGBF transcriptional regulation.

The failure of high ion concentrations to disrupt TFIIB binding by blocking electrostatic interactions was the one of the first indications that acidic activators may utilize more than just charge for PIC contact (Cress and Triezenberg, 1991; Regier *et al.*, 1993). This observation has been repeated with AtTFIIB1 and would seem to fit into the emerging model that hydrophobic patches are most critical for TFIIB interaction and that surrounding charged regions keep the interacting domains soluble and exposed on protein surfaces (Triezenberg, 1995). The use of various hydrophobic agents such as ethylene glycol and detergents to study these interaction surfaces may help discriminate among factors with affinity for TFIIB. Ethylene glycol disrupts YY1 binding (Usheva and Shenk, 1996) but apparently not that of the acidic VP1a domains. The AtTFIIB1 elution profiles from immobilized VP1a and SGBF2 showed slight differences between Triton X-100 and CTAB treatments. Interestingly, SDS and CTAB both have flexible straight-chain hydrocarbon tails of similar length, yet had very different abilities to disrupt AtTFIIB1 binding. The two detergents carry opposite charges in their hydrophilic head groups, and AtTFIIB1 contains the clusters of basic residues typical of TFIIB homologs;

perhaps SDS is more likely to tightly associate with the protein and disrupt interactions compared to the cationic CTAB. Further investigation of the chemical nature of TFIIB interactions would be aided by a series of point mutants within the mapped interaction domains to identify residues directly involved, as would an NMR or co-crystal structure of an activator bound to TFIIB.

The *in vivo* consequences of interactions identified *in vitro* between activator proteins and TFIIB are now the main focus in the field. If cellular TFIIB concentrations are low enough to be limiting for PIC formation, the presence of an activator with TFIIB binding affinity would help localize the factor to activator-specific promoters. This recruitment may be an important means for sequestering TBP, since *in vivo* over-expression of TBP was observed to boost transcription from activator-dependent promoters (see Introduction) suggesting its concentration is indeed limiting. Excess TFIIB, however, had little effect on basal or activated transcription in *Drosophila* and mammalian cells (Colgan *et al.*, 1993; Paal *et al.*, 1997), and over-expression of either TBP or TFIIB did not obviate the need for activators to produce fully enhanced levels of transcription (Choy and Green, 1993; Colgàn *et al.*, 1995; Schmitz *et al.*, 1995; Wampler and Kadonaga, 1992; White *et al.*, 1992). Cellular TFIIB concentrations would therefore seem to not be limiting, yet *in vitro* activator affinity for TFIIB correlates to *in vivo* activation potential (Wu *et al.*, 1996). If these results can be confirmed for a range of ADs with specificity for TFIIB, refinement of models for transcription activation through TFIIB will require consideration of all steps at which regulation might occur and improvement of assays for studying *in vivo* promoter environments. TFIIB recruitment may still be a viable mechanism since it is not clear that raising the overall factor

concentration by transient expression actually increases the amount available to a given promoter. Sub-cellular localization, incorporation into non-productive complexes, increased turnover rates and transcription squelching could all counteract over-expression of TFIIB and have negative effects on a reporter promoter.

Activators may increase the binding affinity of TFIIB for the nascent PIC, which is again a recruitment mechanism but considered from another perspective. The equilibrium dissociation constant for AtTFIIB1 interaction with AtTBP-DNA was 7.1 nM, which is comparable to the K_d values of 2-5 nM observed *in vitro* for TATA binding by most TBP homologs. This represents a higher affinity than was measured for AtTBP binding to the AdMLP TATA (K_d = 34.1 nM) and than was observed for yeast pol II interaction with yTFIIB (K_d = 26 nM) (Bushnell *et al.*, 1996). The values for TBP-TATA binding are in the lower portion of the range of DNA-binding affinities observed for sequence-specific transcription factors. The GAL4-GAL80 complex, for example, has a K_d for its DNA binding site of 2.4 nM (Parthun and Jaehning, 1990) and members of the steroid hormone receptor superfamily showed K_ds of 1-3 nM (Ladias *et al.*, 1992). Most transcription factors, however, exhibit greater affinity for their DNA elements, such as the K_d values observed for Sp1 (0.41-0.53 nM) (Letovsky and Dynan, 1989), Ftz (25 pM) (Florence *et al.*, 1991) and NF-κB (0.1-1.0 pM) (Zabel *et al.*, 1991). Association of these upstream factors with TFIIB would therefore create a high-affinity tether to the promoter DNA, and may introduce cooperativity when the interaction involves TFIIB domains not required for TBP-DNA binding (Pugh, 1996). It is interesting to note that activation also occurs when ADs contact the same TFIIB regions required for TBP-DNA binding, as is the case with acidic activators which bind the TFIIB core domain and have specific

contacts to the E1 and E2 helices (Roberts *et al.*, 1993; Yu *et al.*, 1995). Recent assays of the binding kinetics for acidic AD-TFIIB interactions may offer one explanation. K_d values for TFIIB binding by GAL4 (248 nM), the p65 subunit of NF-κB (23 nM) and the HIV co-activator TAP (250-500 nM) (Paal *et al.*, 1997; Wu *et al.*, 1996; Yu *et al.*, 1995) revealed less affinity than exhibited by AtTFIIB for AtTBP-DNA. These activators may represent highly accessible general binding sites, tethering TFIIB to the promoter and delivering it to a more specific binding site in the TATA region. TBP and DNA contacts would then out-compete the AD for TFIIB, preventing continued association with the activator that might be detrimental for proper PIC formation.

Just as with TFIID binding and subsequent TAF oligomerization, there are probably other steps at which regulators influence TFIIB involvement. One likely candidate is the intra-peptide association that was proposed to produce a conformation change in TFIIB after disruption by VP16 (Roberts and Green, 1994). Oddly, no additional studies of this conformation change have been reported. It might be possible to develop a rapid assay for changes in structure by measuring altered fluorescence emissions, either from internal residues or an attached label, and thus observe the conformation shift in the presence of VP16 or other activators. This technique was used to show that hTBP induces a conformational change in the acidic AD of VP16, and the alterations in fluorescence corresponding to local protein conformations could be measured separately from anisotropy and emission changes that result from formation of a higher molecular weight complex (Shen *et al.*, 1996). AtTFIIB1 contains only two tryptophans, one in the conserved region and another near the C-terminus, and these could serve as convenient fluorescent indicators of local protein conformation (Malencik

and Anderson, 1988; Malencik and Anderson, 1983). A search for point mutations that generate the altered conformation without VP16 being present would identify residues responsible and create a version of AtTFIIB1 incapable of forming the intra-peptide association. Perhaps this change will result in a larger proportion of TFIIB competent for TBP binding; only 25% of the *E. coli* expressed AtTFIIB1 was active for forming the ternary complex. If TFIIB is present in two structural states *in vivo* but only one efficiently interacts with TBP-DNA, activators may induce a conformational shift to the active state and thereby increase transcription. This effect might be measured *in vitro* as a higher specific activity for TFIIB binding to TBP-DNA in the presence of activator (or when using a TFIIB protein defective for formation of the inactive state) rather than as a change in the association or dissociation binding kinetics.

Other mechanisms must also be considered. If TFIIB is consistently found to be a member of the holoenzyme, activators may be recruiting the entire complex instead of the factor alone. The proposed conformation change may be required in the context of the holoenzyme, creating a higher affinity for TFIID and DNA, or exposing activator targets within the assembled PIC. Re-initiation may depend upon activator-stabilized TFIIB not dissociating from the promoter, or TFIIB being held in an active form after pol II exits the PIC. Re-assembly of the holoenzyme after pol II has terminated transcription has yet to be studied and could be directed by activators sequestering required components, producing holoenzyme that is localized to the regulated promoter during assembly. The likelihood of these and other potential models for regulation will no doubt become apparent as more assays for specific steps in the initiation process are developed.

TFIIB is a small but critical component of the PIC, the formation of which is a major node in the web of information management required for viable cellular metabolism. Cell replication, development, adaptation to the environment, and programmed death all require regulated transcription of the proper portions of the genetic library. Curiosity about transcriptional activation and repression in response to the many signals that are carried to the nucleus will drive research deeper into the mechanisms of biochemistry, and may be one key for the synthesis of those mechanisms into a better understanding of biology.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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